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14. ABSTRACT
The mammary gland is made up of several epithelial cell populations including luminal, myoepithelial, stem and progenitor cells. The experiments in this proposal investigate the role of mammary epithelial cell differentiation processes during development and tumorigenesis. Using FACS, mammary epithelial cell (MEC) populations from tumors and wildtype tissue will be investigated for their outgrowth potential or tumorigenic capacity. MECs with enhanced outgrowth or tumorigenic potential will be further characterized by gene expression profiling. We are also developing new mouse models that can be used to isolate viable long-surviving cell populations. Once cell populations with slow turnover have been identified they will be characterized *in vivo* using real-time imaging techniques and isolated by FACS for characterization of their outgrowth capacity and gene expression profiles. We are also interested in studying how genes expressed in different mammary epithelial cell populations affect differentiation and tumorigenesis. For these experiments we are developing techniques for viral transduction and transplantation of primary MECs.

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Table of Contents

INTRODUCTION:

The mammary gland is made up of several epithelial cell populations including luminal, myoepithelial, stem and progenitor cells. The experiments in this proposal investigate the role of mammary epithelial cell differentiation processes during development and tumorigenesis. Using FACS, mammary epithelial cell (MEC) populations from tumors and wildtype tissue will be investigated for their outgrowth potential or tumorigenic capacity. MECs with enhanced outgrowth or tumorigenic potential will be further characterized by gene expression profiling. We are also developing new mouse models that can be used to isolate viable long-surviving cell populations. Once cell populations with slow turnover have been identified they will be characterized *in vivo* using real-time imaging techniques and isolated by FACS for characterization of their outgrowth capacity and gene expression profiles. We are also interested in studying how genes expressed in different mammary epithelial cell populations affect differentiation and tumorigenesis. For these experiments we are developing techniques for viral transduction and transplantation of primary MECs.

BODY:
Task 1. Characterize mammary epithelial cell populations in tumors and the normal gland.
A) Identify cell populations by FACS analysis in mammary epithelial primary cultures isolated from normal and hyperplastic mammary glands and from tumors. ***In progress***

Previous Results: Freshly prepared primary mammary epithelial cells (MECs) that express the Sca-1 cell surface marker display enhanced stem cell activity in functional transplantation assays [1]. This task seeks to characterize the expression of the Sca-1 marker in hyperplastic and tumor tissue. In a collaboration we have analyzed by FACS several mouse mammary gland tumors and hyperplasias for Sca-1 and have reported some of this data [2].

The tumor models that we used included the MMTV-Neu, -Wnt1, and -Polyoma middle T (PyMT) transgenic mice. Primary MECs were isolated from wildtype glands, Neu-induced hyperplasias and tumors, Wnt1 induced hyperplasias and tumors, and PyMT-induced tumors and were FACS analyzed for Sca-1. Immunohistochemisty of MEC differentiation markers was also performed on the same mouse models in this collaboration. These data suggest that several mouse models of mammary tumorigenesis can be separated into "progenitor" type tumors, that display myoepithelial, luminal and Sca-1 cell markers, or "luminal" type that are restricted for expression of the luminal epithelial cell marker keratin-8.

Another goal of this project is to further refine the Sca-1 cell population by identifying markers that are either coexpressed or absent on Sca-1 MECs. If a cell surface marker can be identified on Sca-1 cells that marks (or is absent) on a fraction of those cells then further enrichment of MEC stem cell activity may be achieved. Results from screening wildtype tissue suggest that CD24 and Sca-1 can be resolved as distinct cell populations representing Sca-1 alone, Sca-1/CD24 double positive, CD24 alone and double negative cell populations. In freshly prepared wildtype mammary glands Sca-1 and CD24 are each expressed on about 30% of MECs.. Using CD24 as a second marker Sca-1 cells can be refined into Sca-1 alone cells representing about 15-20% of total freshly prepared MECs and Sca-1/CD24 double positive cells representing about 5-10% of MECs. Since the MEC Sca-1 population has enhanced outgrowth capacity the ability of CD24 to further resolve the Sca-1 population could provide a tool for further enrichment of MEC stem cells. Subtasks *b* and *c* below characterize marker expression and outgrowth potential of the Sca-1 and CD24 sorted cells. Additionally, CD24 is a small GPI-anchored mucin-like protein that can bind to P-selectin expressed on activated endothelial cells and may mediate rolling of breast carcinoma cells along blood vessels [3]. This function may influence breast carcinoma metastasis. Thus, we screened mouse mammary tumor models for Sca-1 and CD24. We found that Sca-1 expression was dependent on the oncogene that induced

4

the tumor while CD24 expression increased in all mouse tumor models. Additionally, the percentage of Sca-1/CD24 double positive cells changed between tumors and hyperplasias and between tumor models. For example, in Wnt-1 induced hyperplasias about 70% of CD24 positive cells also express Sca-1, however, in tumors the number of double positive cells decrease to about 50%.

New Results: As described above we previously observed that Wnt-1 and PyMT induced tumors exhibit marked differences in expression of keratin-6, keratin-14 and Sca-1. Since WAP-MMP3 induced tumors have many histological characteristics that are similar to MMTV-Wnt-1 induced tumors we examined archived tumor samples from WAP-MMP3 transgenic mice for keratin-6 expression. We observed that these WAP-MMP3 tumors also express abundant levels keratin-6 with regions of squamous metaplasia similar to that observed in MMTV-Wnt1 induced tumors (Figure 1). We are interested in using the viral transduction technique described in Task 5 to further study potential associations of MMP3 and Wnt signaling.

We have characterized the expression of a number of cell surface markers in Wnt-1 induced hyperplasias, tumors and in normal mature mammary epithelium using 2 and 3 color FACS analysis (data not shown). We have also optimized the procedure used to produce single cell suspensions of mammary epithelium. The new procedure uses two steps of enzymatic digestion and differential centrifugation resulting in a very pure (>90% keratin 14/18 positive) single cell suspension. Figure 6B shows keratin-18 staining of the plated primary cells using this isolation technique and demonstrates that >90% of the cells are K18 positive. These results are also consistent with FACS analysis of K18 expression in these cell preparations. The decrease in contamination of blood and stromal cells using this procedure increases the purity of mammary epithelium and produces more consistent results by FACS analyses. We have used this procedure and analyzed the expression of the cell surface markers Sca-1, CD24, CD49F, Thy1.1, and CD29. Our FACS data demonstrate that primary MECs cells that are Sca-1^{low} / CD24^{high} are also Thy1.1^{low}, CD49f^{high} and CD29^{high} (data not shown). A number of interesting populations have also been observed in Wnt-1 induced hyperplasias and tumors and will be further analyzed in sub-tasks *b* and *c* described below.

B) Characterize gene expression in sorted cell populations by RT-PCR and Western blot analyses. *In progress*

Previous Results: We examined the expression by immunofluorescence of K14 (myoepithelial marker), K8 (luminal epithelial marker) and K6 (putative progenitor marker) in sorted Sca-1 cells from wildtype and MMTV-Wnt1 induced hyperplasias and tumors [2] . For most of these experiments MECs were cultured for 5-7 days on plastic, trypsinized and FACS analyzed. Culturing the primary MECs prior to FACS analysis allows the cells to more easily disaggregate and resulted in increased sorting efficiency. However, culturing prior to sorting also altered cell surface marker profiles when compared with sorting fresh MECs. We observed that in cultured MECs Sca-1 alone cells were negative for the keratin markers K6, K8 and K14. However, Sca-1^{high}/CD24^{high} and Sca-1^{low}/CD24^{high} double positive cells were positive for all three markers. The most K6 positive cells were observed in the Sca-1^{low}/CD24^{high}.

New Results: We are currently performing microarray experiments to identify genes expressed in several of the mammary epithelial cell populations identified in Task 1a. We have developed a culturing and purification method that enriches for cells with potent outgrowth potential as in Task 5 and we have identified a number of interesting candidate genes including cell surface markers that are expressed on these cells. We have also identified a number of cell cycle

5

regulators, matrix proteins, transcription factors and growth factor genes that are differentially expressed on cells with enhanced outgrowth potential as compared to cells with limited outgrowth potential (data not shown). Our microarray analysis has also confirmed the differential regulation of Sca-1 and CD24 as described in our previous results.

C) Transplant enriched epithelial cell populations into cleared fat pads. ***In progress***

Previous Results: We have transplanted FACS sorted and enriched cell populations from MMTV-PyMT tumors and wildtype mouse mammary glands into the cleared fat pads of syngeneic mice. These data suggest that the Sca-1 alone cell population in cultured MECs is not enriched for stem cell activity or tumorigenic potential. Rather, the Sca-1/CD24 double positive population displays both tumorigenic potential and outgrowth capacity when isolated from MMTV-PyMT and wildtype tissue, respectively. Interestingly, in wildtype tissue the Sca-1^{low}/CD24 double positive cells contained about 2.5-times more K6 cells than Sca-1^{high}/CD24 and they also exhibited the best transplantation take-rate (100%) and the most growth in the fat pad (70% of the transplants had full outgrowth) (Figure 2). When Sca-1/CD24 cells were transplanted from MMTV-PyMT induced tumors the Sca-1 alone cells displayed very poor tumor growth. Both Sca-1^{high}/CD24^{high} and Sca-1^{low}/CD24^{high} sorted cell population exhibited tumorigenic potential.

New Results: We have previously determined that FACS sorted Sca-1^{low} /CD24 ^{high} cells are enriched for outgrowth potential when using a cleared fat pad transplantation assay of sorted populations (Figure 2). We have made new progress in developing a culturing method that specifically expands this population. Using this culture method we have been able to enrich for cells with enhanced outgrowth potential without prior sorting (Figure 3). We also observed that that cells cultured using our method are enriched for Sca-1^{low} /CD24 ^{high} cells (Figure 4). We are currently performing microarray and FACS analyses of the cultured cells to further characterize them. We are also currently comparing the limited dilution outgrowth potential of the enriched, depleted and the untreated cultured cells. We anticipate these results will be completed in 4-6 months.

D) Analyze Sca-1 localization in Sca-1^{GFP}/ MMTV-Neu and Sca-1^{GFP}/ MMTV-Wnt1 mouse mammary tumors using real-time two-photon confocal imaging. ***Completed***

Previous Results: We have optimized a technique to visualize in real-time cell-cell interactions in the mammary gland of living mice. A major portion of this task has been to develop methods to anesthetize mice for 4-12 hours while maintaining body temperature, hydration and respiration and develop surgical techniques to expose and immobilize the mammary gland without rupturing blood vessels. Additionally, we optimized the microscope settings and optics to perform this technique. This imaging technique provides insight into the cell types that express Sca-1, their location in the tissue and interaction with other cells. For these experiments we use mice that have EGFP knocked-in to the Sca-1 locus to visualize Sca-1 expressing cells in the mammary gland. Sca-1 is also expressed in the blood vessels and leukocytes so it provides a nice imaging tool to visualize tumor cell interactions with the vasculature. We have bred MMTV-PyMT and MMTV-Wnt-1 to Sca1-EGFP mice allowing us to visualize Sca-1 positive cells in hyperplasias and tumors. We have decided to use MMTV-PyMT mice instead of MMTV-Neu (as proposed) since MMTV-PyMT and –Neu both have similar "luminal" tumor phenotypes but PyMT induced tumors arise much faster.

By FACS analyses about 10% of cells isolated from MMTV-PyMT tumors were positive for Sca-1. We were interested in determining if the low number of Sca-1 cells in the PyMT

tumors represent a "tumor stem cell" that could give rise to luminal cell types. However, when we imaged Sca1-EGFP/MMTV-PyMT tumors we observed that the tumor epithelium is negative for EGFP. All EGFP positive cells in the PyMT tumor appeared to be either endothelial cells or infiltrating leukocytes. This imaging suggests that the 10% Sca-1 cells detected in the PyMT induced tumors by FACS most likely represent blood vessels and leukocytes isolated during primary culture preparation of the MECs. By contrast, the MMTV-Wnt-1 induced tumors are highly positive for Sca-1 expression and exhibit expression of EGFP in the tumor epithelium. Using this imaging technique we have been able to confirm that Sca-1 is differently expressed in tumor epithelium and to visualize interactions of tumor cells with infiltrating leukocytes in both MMTV-PyMT and MMTV-Wnt-1 mouse models.

New Results: In August 2005 we moved our mouse lines into a new barrier-type animal housing facility requiring us to rederive a number of our mouse lines. This has resulted in a significant reduction of available mice for performing these experiments. Additionally, during the past several months we purchased and optimized new imaging equipment specifically designed for our *in vivo* imaging experiments. We plan on performing more *in vivo* imaging experiments in the next year however due to these delays *Task 1d* has been removed from my new statement of work (see appendix).

E) Isolate and transplant Sca-1 and CD24 cell populations from Wnt-1 tumors and hyperplasias to determine their tumorigenic potential. *In progress*

New Results: In collaboration with A. Welm and M. Bishop we are currently performing a number of limited dilution transplantations of Wnt-1 and PyMT tumor cells into syngeneic mice to determine the number of cells required to form tumors. Both models appear to have different requirements of transplanted cells to induce tumor formation. Between 1,000 and 10,000 cells isolated from MMTV-Wnt-1 induced tumors are required to form tumors upon transplantation into cleared fat pads of syngeneic mice (Figure 5). However, only about 100-200 cells from MMTV-PyMT induced tumors are required to form tumors upon transplantation. These experiments will determine the number of transplanted cells required to form tumors without prior sorting and provide a comparison of tumorigenic capacity for the sorted populations. We will be sorting and transplanting several of the cell populations identified in Task 1a during the next 12 months.

Task 1
Discussion: This task seeks to characterize the Sca-1 marker and identify novel markers in the normal mammary gland and tumors that can be used to characterize mammary epithelial cell progenitors during tumorigenic progression. We have found that several mouse mammary tumor models can be divided into "progenitor" and "luminal" phenotypes. Progenitor tumor phenotypes contain cells that express myoepithelial and luminal cell types and often exhibit some degree of cellular organization. In many of these tumors the K14 (myoepithelial) and K8 (luminal) positive cells are structured into distinct concentric layers suggesting organizational signals are maintained. Putative progenitor cell markers such as keratin-6 and Sca-1 are also highly expressed in these tumors and their initial hyperplasias. Interestingly, progenitor type tumors are mainly observed from oncogenes in the Wnt/β-catenin pathway including Wnt-1, Myc, and β-catenin. Furthemore, squamous metaplasia is often observed in regions of these tumors. In contrast, luminal tumor phenotypes are mainly observed with receptor tyrosine kinase pathway oncogenes such as Neu, Ras and PyMT. These tumors are poorly organized and express almost exclusively the K8 luminal epithelial marker. These data suggest that specific oncogenic pathways expand a particular cell type that can give rise to the tumor mass. Since tumors result

7

from clonal expansion the "progenitor tumor" phenotype must be derived from a cell type that can give rise to both luminal and myopeptithelial cells while "luminal tumors" are somehow restricted to a luminal-type differentiation. Further studies will determine whether progenitor type hyperplasias have enhanced stem cell activity when assayed by limited dilution transplantation.

We have also identified a new marker, CD24, expressed on a subset of Sca-1 MECs. We have further characterized cells that express this marker in Task1*b* and *c*. We have examined the expression of CD24 and Sca-1 in mouse mammary tumor models and we have performed transplantation and IHC on sorted Sca-1/ CD24 cell populations. Several observations and technical issues have been raised from these experiments that must be addressed in further experiments. For example, the expression of Sca-1 increases from about 30% positive total cells in freshly prepared primary MECs to about 80% in MECs cultured on plastic. Furthermore, many immortalized MEC cell lines such as EpH4 and SCP2 cells also express high levels of Sca-1. However, cells that are cultured in Matrigel express Sca-1 in only about 15-20% of epithelial cells suggesting that culturing conditions are critical to maintain a restricted expression pattern for Sca-1. Interestingly, even primary MECs of PyMT tumors cultured on plastic are highly positive for Sca-1 while by *in vivo* imaging and FACS of freshly prepared MECs the tumor epithelium is negative for Sca-1. Thus, an increase in Sca-1 expression when culturing on plastic may be an artifact of the culturing conditions. We now believe, that during culturing, a subset of mammary epithelial cells undergoes epithelial-to-mesenchymal transition (EMT) resulting in the loss of CD24 expression while Sca-1 expression is maintained or gained. The data from Task1 *b* and *c* suggest the cells that expressed low levels of Sca-1 and that were positive for CD24 were also enriched for K6 expression and correlated with better outgrowth of normal mammary epithelium than cells expressing high levels of Sca-1. The Sca-1 alone cells (CD24 negative) gave very poor outgrowth and were mostly negative for K6, K14 and K8. Thus, it appears when cultured on plastic, low levels of Sca-1 correlate with greater stem cell activity (Figures 2, 3 and 4).

During the past year we have analyzed by FACS a number of cell populations in Wnt-1 hyperplasias and tumors and in wildtype mammary glands. The cell surface markers we are using include CD49f, CD24, CD29, Thy1.1, and Sca-1. Using these markers we have been able to further resolve distinct populations that overlap with Sca-1^{low} / CD24^{High} that we previously observed contained stem cell activity or tumorigenic potential. These cell populations are currently being transplanted into syngeneic mice to determine their tumorigenic potential (when isolated from tumors) or outgrowth potential (when transplanted from wildtype mice).

Task 2. Generate Sca-rtTA-IRES-tTS and CAG-rtTA-IRES-tTS mice. *Completed*

Previous Results: The goal of these Tasks is to develop a mouse model that can be used to visualize and isolate long surviving/slow turnover cells in the mammary gland. This method is based on data that stem cells have slow turnover in several organs including the mammary gland. Cells that slowly turnover can be identified by using stabilized fluorescent proteins that can be transiently transcribed (pulse) followed by a period when no new protein is generated (chase). During the chase period cells that are actively proliferating or cells that undergo apoptosis will not be labeled leaving a fluorescent quiescent cell population. These cells can be isolated by FACS or visualized in the mammary gland using the imaging techniques described in Task1*c*. To perform these experiments we have obtained the stabilized fluorescent proteins H2B-EGFP and H2B-mRFP, cloned them under the control of the Tet-responsive promoter (TRE) and have generated transgenic mice. H2B-EGFP and H2B-mRFP are histone-2B proteins fused with green and red fluorescent proteins that are highly stabilized. We have also generated Chicken-actin

(CAG)-rtTA-IRES-tTS transgenic mice that express rtTA and tTS in all tissue. These CAG-rtTA-IRES-tTS mice will be bred with the TRE-H2B-EGFP and TRE-H2B-mRFP mice to establish bigenic strains. These bigenic mice will be treated with dox transiently and then chased for 1-4 weeks without dox to allow isolation and visualization of slow turnover cells in the mammary gland. Additionally, once the Sca1-rtTA-IRES-tTS mice have been established we can perform the same experiment using bigenics of Sca1-rtTA-IRES-tTS and TRE-H2B-EGFP or TRE-H2B -mRFP to specifically isolate Sca-1 cells with slow turnover. At this time we have generated CAG-rtTA-IRES-tTS, TRE-H2B-EGFP and TRE-H2B-mRFP and are currently characterizing expression of the transgenes.

Upon testing a rtTA construct we determined that rtTA had high dox-independent transcription activity. We found that in the presence of dox only a 4-fold increase in transcriptional activity was observed mainly due to high background activity. To reduce the dox-independent rtTA activity we subcloned an rtTA-IRES-tTS insert into the Sca-1 targeting plasmid. This construct expresses the rtTA and tTS together under the Sca-1 promoter. tTS is a variant of tTA that contains a KRAB-AB repressor domain in place of a VP16 transaction domain and represses background rtTA transcriptional activity by binding to the TRE in the absence of dox. In the presence of dox tTS comes off the TRE allowing rtTA to bind and activate transcription. We observed a significant reduction of background transcription when using the rtTA-IRES-tTS construct allowing for a 270-fold increase in transcriptional activity over uninduced cells. This increase in dox induced transcriptional activity results from a significant reduction of background transcriptional activity by about 100-fold. Thus, we repeated the transfection of ES cells with the new Sca1-rtTA-IRES-tTS targeting construct and we now have 160 clones that are currently being screened for proper targeting. Based on our collaborators experience that this construct is about 2-3% efficient at generating properly targeted ES cell clones we anticipate about 3-4 positive clones will be established. Once we have identified properly targeted ES clones we will perform blastocyst injections and establish mouse lines.

New Results: Over the past year we generated and characterized several transgenic mouse lines with the Chicken-beta-actin (CAG) promoter driving rtTA-IRES-tTS. We also generated several TRE-H2BEGFP and TRE-H2BmRFP transgenic lines that we planned on breeding with the CAG-rtTA-IRES-tTS mice. We initially characterized the CAG-rtTA-IRES-tTS founder lines by isolating tail fibroblasts and infecting them with a retrovirus containing the TRE promoter driving EGFP or mRFP. Likewise, we characterized the TRE-H2BEGFP and TRE-H2BmRFP transgenic lines by infecting tail fibroblast with a retrovirus containing tTA. We observed that infected fibroblasts from several of these transgenic lines did express, in an inducible manner, the EGFP or mRFP. We then bred some of the CAG-rtTA-IRES-tTS expressing mouse lines with the TRE-H2BEGFP and TRE-H2BmRFP expressing lines and treated mice with Dox pellets for several weeks. When we analyzed the mice we did not observe EGFP or mRFP expression in the Dox treated bigenic lines. We also isolated tail fibroblasts from the bigenic lines and could not detect induction of EGFP or mRFP in the cultured fibroblasts grown in the presence of dox. Upon discussing these results with another researcher here at UCSF whom attempted to also develop rtTA / tTS bigenic mice (using two separate constructs co-injected into oocytes) I was told that they were not successful at establishing their mouse lines. Our initial experiments using cell culture studies (described in "previous results") suggested that rtTA and tTS are compatible and the addition of tTS is required to reduce dox-independent expression of TRE-H2B-EGFP. However, the cells transfected in these experiments probably had multiple integrations of the TRE-H2BEGFP and CAG-rtTA-IRES-tTS resulting in less sensitivity to the presence of tTS because of high expression levels of rtTA. Our transgenic mice most likely had

few copies of the transgene and reduced expression of rtTA resulting in tTS repressing rtTA's transactivation capacity.

Since the rtTA-IRES-tTS cassette was used in both the CAG-rtTA-IRES-tTS and Sca1-rtTA-IRES-tTS constructs and it is likely that a similar problem will arise from tTS interference with rtTA we have decided not to pursue the generation of the Sca1-rtTA-IRES-tTS knockin mice using our previously selected ES cell lines. The goal of Task 2 is to develop mouse models that express transgenes in different populations of mammary epithelial cells. The Sca-1 promoter would express transgenes in progenitor cells while the CAG promoter would drive expression in all cells of the mammary epithelium. We feel our best approach is to develop a Sca-1-tTA or Sca-1-rtTA BAC transgenic in collaboration with Dr. Yi Li at Baylor College of Medicine or acquire a Keratin-6-tTA mouse line for expressing transgenes in mammary epithelial progenitors. The alternative strategy for the CAG-rtTA-IRES-tTS transgenic line is described below in Task 5.

Task 3. Generate tetracycline responsive (TRE) H2B-EGFP and H2B-mRFP mice. Completed

New Results: This task has been completed and described above in Task 2.

Task 4. Isolate and characterize H2B-EGFP and H2B-mRFP long-term label retaining cells (LRCs). In progress

New Results: The TRE-H2BEGFP mice (described in Task 2) will be used as donors for primary MECs and will be infected with a virus expressing rtTA. The infected cells will be transplanted into the fat pads of syngeneic mice. About 4 weeks after the transplantation the mice will be treated with dox for 1 week and then the outgrowths will be removed about 2-4 weeks later. The cells that remain labeled with EGFP will be analyzed by confocal imaging and isolated by FACS. During the past year we have generated the TRE-H2BEGFP transgenic mice, developed the rtTA viral constructs and optimized the infection technique (described in Task 5 below). We will be performing these experiments during the next few months.

Task 5. Optimize the retroviral/lentiviral infection and transplantation of primary MECs using the CAG-rtTA-IRES-tTS transgenic line as donors.

In collaboration with Dr. Peter Dijkraaf we are optimizing a method to efficiently test gene function in an *in vivo* model system. We are interested in using retroviral and lentiviral methods to introduce genes into mouse mammary primary cultures for studying gene function *in vitro* and *in vivo*. We believe that using this methodology will allow for more efficient use of mice, reduce our animal housing requirements and screen more genes in a physiologically normal environment.

We have performed a number of techniques to optimize the method published by A.Welm, et.al [4]. We have observed that the murine stem cell virus (MSCV) based infection technique developed by A.Welm's works well to study genes that provide a growth promoting affect to the transduced cells. However, genes with growth neutral affects (such as EGFP) have limited outgrowth success most likely as a result from competition of uninfected stem cells. For example, using the MSCV based method as described by A. Welm about 70-90% of the ductal epithelium in each transplant is positive for retroviral infection when c-Myc was used as the transgene. However, when using the control virus that contains only EGFP, only 10-20% of transplants contained any ducts positive for retroviral infection. Additionally, in the transplants that are positive for retroviral infection only 1 or 2 ducts out of the 20-50 ducts present in the outgrowth show EGFP expression (Figure 6 and data not shown). Since viral titers are about the

same for the c-myc and control EGFP viruses (the overall infection of the primary culture cells prior to transplantation is about 20-30% for both viruses) these results suggest that this infection method targets only a fraction of the stem cells. Therefore, unless a gene, such as an oncogene like c-myc, provides a growth advantage the infected stem cells are out competed by wildtype stem cells during the outgrowth.

We have performed a number of experiments to optimize the infection technique. We experimented with different retroviruses and lentiviruses, use of selectable markers, insertion of RNA stability elements and insulators in the viral backbone, and supplementation of culture media with growth factors to optimize the infection technique [5]. Additionally, since proliferation is required for retroviral infection we also analyzed proliferation rates during culturing of the primary MECs to identify optimal infection times (Figure 7). During these experiments we have been able to create a method to culture and enrich for cells with enhanced outgrowth potential using a technique that does not require cell sorting (Figure 3 and 4). The enrichment technique selects for cells that are Sca-1^{low} / CD24^{high} (Figure 4). Mammary epithelial primary cells enriched by this technique were easily transduced with retroviruses and lentiviruses (60-80% of primary MECs were infected) and efficient growth of transplanted cells was observed (Figure 8). We have observed that greater than 90% of the transplants exhibited ductal epithelium that was positive for infection. Additionally, about 80-100% of the mammary epithelium in each positive outgrowth expresses our reporter genes (EGFP , ZsGreen or mRFP). These data suggests that this infection method efficiently targets stem cells since these experiments were performed with non-growth-promoting reporter genes. This procedure will be used in Tasks 3-6.

A) Generate Tet-regulated retro- and lentiviral vectors. **Completed**

New Results: We have developed several constitutive and Tet-regulated viral constructs (Figure 9).

B) Test vectors using wildtype FVB donors. **In progress**

New Results: We have tested several constitutive viral vectors and we currently testing the inducible viruses. We anticipate that these experiments will be complete and published within a year

C) Test vectors using CAG-rtTA-IRES-tTS donors. **In progress**

New Results: Due to the problems with the CAG-rtTA-IRES-tTS transgenic mice we will not use these mice as donors of primary MECs for these experiments. Instead of using the CAG-rtTA-IRES-tTS mice as donors we are co-infecting wildtype FVB/N primary MECs with an rtTA expressing virus and TRE-H2BEGFP virus (Figure 6). These experiments are currently being performed.

Task 6. Use the optimized technique for infection of MECs to study the role of Kuzbanian in mammary gland development. In progress

New Results: These experiments will be performed in the next year.

TRAINING
During the past year I have attended two scientific meetings including the Era of Hope meeting in Philadelphia, PA and the International Society for Stem Cell Research meeting in San Francisco, CA. Myself and several other postdoctoral fellows at UCSF have recently started an informal seminar series at UCSF to discuss our research on mouse models of mammary cancer. These meetings are held twice a month. I also participated in laboratory meetings, attended local seminars and trained rotating students.

KEY RESEARCH ACCOMPLISHMENTS:
-Identified several cell surface markers with overlapping Sca-1^{low} / CD24^{high} expression that can be used to further enrich for stem cell and tumorigenic capacity.
-Generated CAG-rtTA-IRES-tTS, TRE-H2B-EGFP and TRE-H2B-mRFP transgenic mice.
-Developed and tested several retrovirus and lentivirus constructs.
-Developed a culturing technique that expands Sca-1^{low} / CD24^{high} primary MECs and does not require FACS.
-Optimized the viral infection method of primary MECs that significantly increases the efficiency of the method.

REPORTABLE OUTCOMES:
Welm AL, Kim S, **Welm BE**, Bishop JM.
MET and MYC cooperate in mammary tumorigenesis.
Proc Natl Acad Sci U S A. 2005 Mar 22;102(12):4324-9.

Bryan Welm, Yi Li, and Zena Werb.
Characterization of Mamamry Epithelial Cell Populations by FACS and Real-time Imaging.
Platform Presentation. Era of Hope Meeting. Philadelphia, PA (2005).

Li Y, **Welm B**, Podsypanina K, Huang S, Chamorro M, Zhang X, Rowlands T, Egeblad M, Cowin P, Werb Z, Tan LK, Rosen JM, Varmus HE.
Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells.
Proc Natl Acad Sci U S A. 2003 Dec 23;100(26):15853-8. Epub 2003 Dec 10.

Bryan E. Welm, Mikala Egeblad, Max Krummel and Zena Werb. Real-time imaging of mammary carcinoma progression. Platform Presentation. AACR-Advances in Beast Cancer Research Conference. Huntington Beach, CA (2003)

CONCLUSIONS:
Understanding the etiological events that can give rise to tumors is critical for prevention, early diagnoses and treatment of breast cancer. The studies being performed here will elucidate cell populations that are targeted by specific oncogenic pathways. We have observed that tumors can be divided into progenitor and luminal phenotypes suggesting that oncogenic events can target specific differentiation pathways. These data suggest that diverse mechanisms and cell populations can contribute to tumorigenesis. The results of this work may aid in the development of new therapeutics that target specific cell populations or differentiation pathways. Our research to date has identified CD24 was as a marker expressed on a subset of Sca-1 MECs that can be used to separate cell populations with enhanced outgrowth potential. The significance of identifying markers coexpressed on Sca-1 cells allows us to further enrich the Sca-1 cell population for stem cell activity. By sorting with Sca-1 and CD24 markers we have characterized several MEC populations for differentiation factors and outgrowth potential. Once a highly

enriched stem cell population is identified then future studies can address how these cells contribute to mammary tumorigenesis.

We are also in the process of developing several mouse models and methods that can be used for our studies to characterize the role of MECs stem cells in tumorigenesis. These mouse models will allow us to enrich for stem cells, observe cell interactions *in vivo* and target gene expression to specific cell populations. We have optimized techniques to visualize fluorescently labeled cells in the mammary glands of living mice. Additionally, transgenic lines that express stabilized green and red fluorescent proteins under the control of the tet-responsive promoter. We have also developed a number of useful viral constructs and developed an efficient primary MEC transduction and transplantation technique that can used to study the effects of genes on mammary development and tumorigenesis. These mice and methods will be useful not only for our studies but should also benefit many investigators in the mammary gland biology research community.

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2.   Li, Y., et al., *Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15853-8.
3.   Aigner, S., et al., *CD24 mediates rolling of breast carcinoma cells on P-selectin.* Faseb J, 1998. **12**(12): p. 1241-51.
4.   Welm, A.L., et al., *MET and MYC cooperate in mammary tumorigenesis.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4324-9.
5.   Ventura, A., et al., *Cre-lox-regulated conditional RNA interference from transgenes.* Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10380-5.

Statement of Work

The Analysis of Cell Population Dynamics in Mammary Gland Development and Tumorigenesis

Task 1. Characterize mammary epithelial cell populations in tumors and the normal gland.

Isolate and transplant Sca-1 and CD24 cell populations from Wnt-1 tumors and hyperplasias to determine their tumorigenic potential (Months 18-36).

Analyze the transplanted tumor pathology and the expression of molecular markers (Months 24-36).

Task 2. Generate Sca-rtTA-IRES-tTS and CAG-rtTA-IRES-tTS mice.

Transfect ES cells with Sca-rtTA-IRES-tTS and select for stable integration (Months 12-18).

Screen Sca-rtTA-IRES-tTS cells for proper targeting (Months 18-20).

Inject blastocycts with properly targeted ES cells (Months 20-22).

Breed chimeras and establish founder lines (Months 22-28).

Generate CAG-rtTA-IRES-tTS transgenic lines (Months 12-24).

Characterize founder CAG-rtTA-IRES-tTS transgenic mice (Months 18-28).

Task 3. Generate tetracycline responsive (TRE) H2B-EGFP and H2B-RFP mice.

Establish and characterize transgenic founder lines (Months 12-28).

Breed into CAG-rtTA-IRES-tTS line (Months 18-26)

Breed into the Sca-rtTA-IRES-tTS line (Months 28-36).

Task 4. Isolate and characterize H2B-EGFP and H2B-mRFP long-term label retaining cells (LRCs).

Breed CAG-rtTA-IRES-tTS mice with TRE-H2B-mRFP and TRE-EGFP mice (Months 18-36).

Localize LRCs in the mammary gland by *in vivo* imaging and use immunofluorescence to colocalize LRCs with differentiation markers (*i.e.* progesterone receptor, keratin-6, keratin-8, keratin-14, smooth muscle alpha-actin) (Months 20-36).

Isolate LRCs and perform limited dilution transplantation (Months 24-36).

Isolate LRCs by FACS cell sorting and perform microarray and expression analyses (Months 24-36).

*Task 5.*Optimize the retroviral/lentiviral infection and transplantation of primary MECs using the CAG-rtTA-IRES-tTS transgenic line as donors.

Generate Tet-regulated retro- and lentiviral vectors (Months 16-24).

Test vectors using wildtype FVB donors (Months 18-24)

Test vectors using CAG-rtTA-IRES-tTS donors (Months 22-26).

*Task 6.*Use the optimized technique for infection of MECs to study the role of Kuzbanian in mammary gland development.

Generate TRE-kuzbanian retrovirus vectors (Months 22-24).

Infect primary MECs from CAG-rtTA-IRES-tTS transgenic mice and transplant transduced cells (Months 24-36).

Analyze phenotype of kuzbanian gene expression on mammary development and tumorigenesis (Months 24-36).

CHARACTERIZATION OF MAMMARY EPITHELIAL CELL POPULATIONS BY FACS AND REAL-TIME IMAGING

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Alterations in the stem/progenitor cell niche may occur during neoplastic transformation of the breast and play a role in tumorigenesis and metastasis. To study the role of mammary stem cells in tumorigenesis we are developing mouse models that will allow inducible gene expression in different luminal cell populations. We have previously identified a population of cells in the mammary gland that express the cell surface marker Sca-1 and when these cells were enriched they exhibit potent regenerative capability in cleared fat pads. The reverse tetracycline-responsive transactivator (rtTA) has been knocked-in to the Sca-1 locus to study how oncogene expression in the Sca-1 expressing subset of mammary epithelium affects tumorigenesis and metastasis.

We have also performed FACS analyses to further define cellular populations in the mammary gland and tumors that have both normal and tumorigenic outgrowth potential. We identified several unique markers with overlapping Sca-1 expression patterns that exhibit altered expression profiles in Wnt-1, Neu, and polyoma middle-T (PyMT) induced mammary tumors. We observed by florescence activated cell sorting (FACS) that greater than 50% of the Wnt-1 induced tumor epithelium is composed of Sca-1 expressing cells while in PyMT and Neu induced tumor epithelium less than 10% of cells express Sca-1. We were interested in determining if the low number of Sca-1 cells in the PyMT tumors represent a "tumor stem cell" that could give rise to luminal cell types. However, when we performed real-time imaging of Sca1-EGFP/MMTV-PyMT tumors we observed that the tumor epithelium is negative for EGFP. All EGFP positive cells in the PyMT tumor appeared to be either endothelial cells or infiltrating leukocytes. This imaging suggests that the 10% Sca-1 cells observed in the PyMT induced tumors by FACS most likely represents blood vessels and leukocytes isolated during primary culture preparation of the MECs. By contrast, the MMTV-Wnt-1 induced tumors are highly positive for Sca-1 expression and exhibit expression of EGFP in the tumor epithelium. Furthermore, Wnt-1 tumors express both the luminal marker keratin-8 and myoepithelial marker keratin-14 in addition to keratin-6 while Neu and PyMT induced tumors are mostly limited to expression of keratin-8. Using this imaging technique we have been able to confirm that Sca-1 is differently expressed in tumor epithelium and to visualize interactions of tumor cells with infiltrating leukocytes in both MMTV-PyMT and MMTV-Wnt-1 mouse models. These data demonstrate that the mammary epithelium displays significant heterogeneity and further suggests that specific epithelial cell populations contribute pathogenesis of the mammary gland. This research will ellucidate how mammary epithelial cell populations contribute to tumorigenesis and may provide insight for targeting theraputics to the most potent tumorigenic cell types.

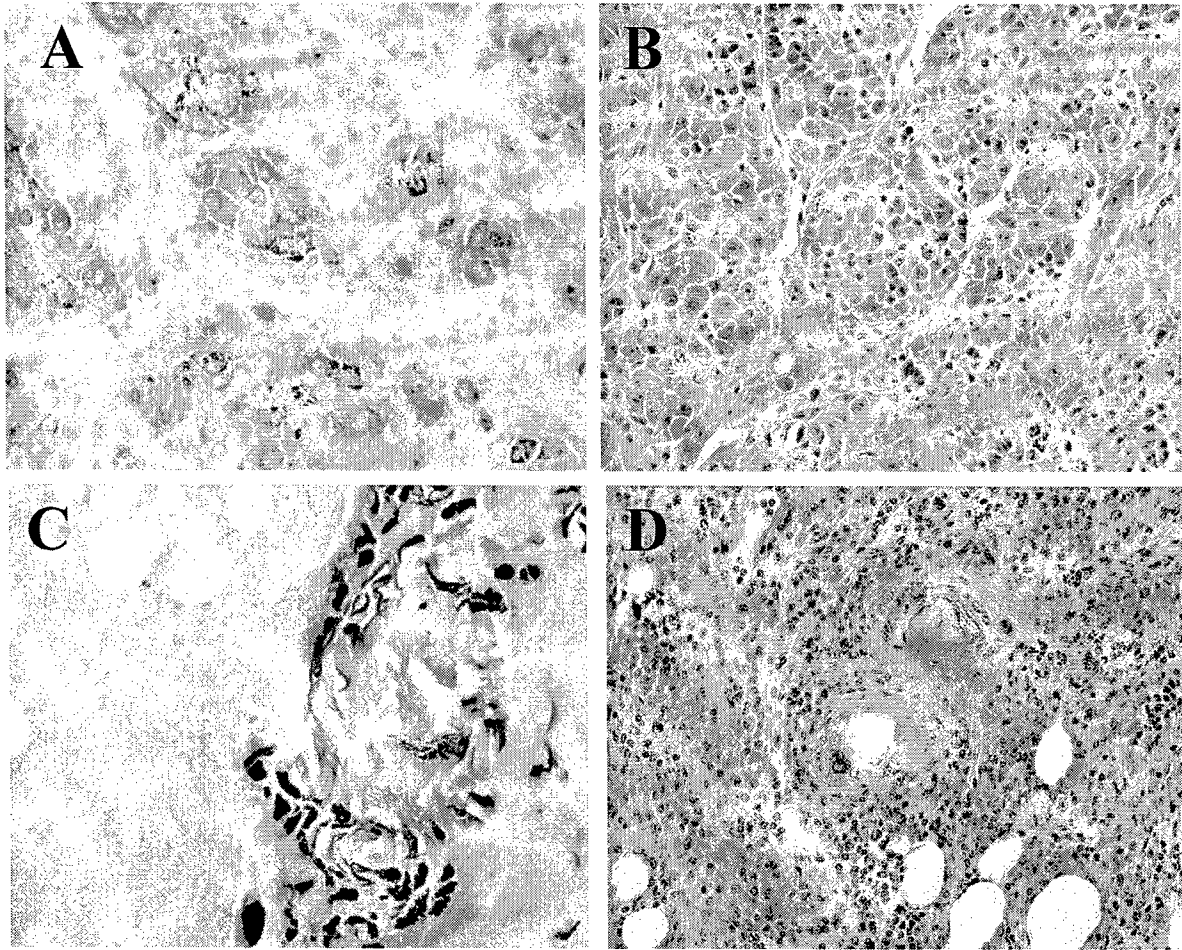# WAP-MMP3 Induced Tumors Express Keratin-6

Figure 1. WAP-MMP3 induced tumors have increased keratin-6 expression. Keratin-6 immunohistochemistry of WAP-MMP3 tumors (A and C). H&E staining of WAP-MMP3 tumors (B and D). Keratin-6 immunoreactivity is observed in focal regions of tumors (A and C) and is lost in poorly differentiated regions (data not shown). These tumors also show keratin-14, smooth muscle alpha-actin and keratin-18 immunoreactivity (data not shown). Squamous metaplasia is also observed in WAP-MMP3 induced tumors (C and D). This histology is similar to that observed in MMTV-Wnt-1 and MMTV-activated beta-catenin induced tumors. Magnification is 20x.

Sca-1 ^{Low} /CD24 ^{High} Cells Have Enhanced Outgrowth Potential
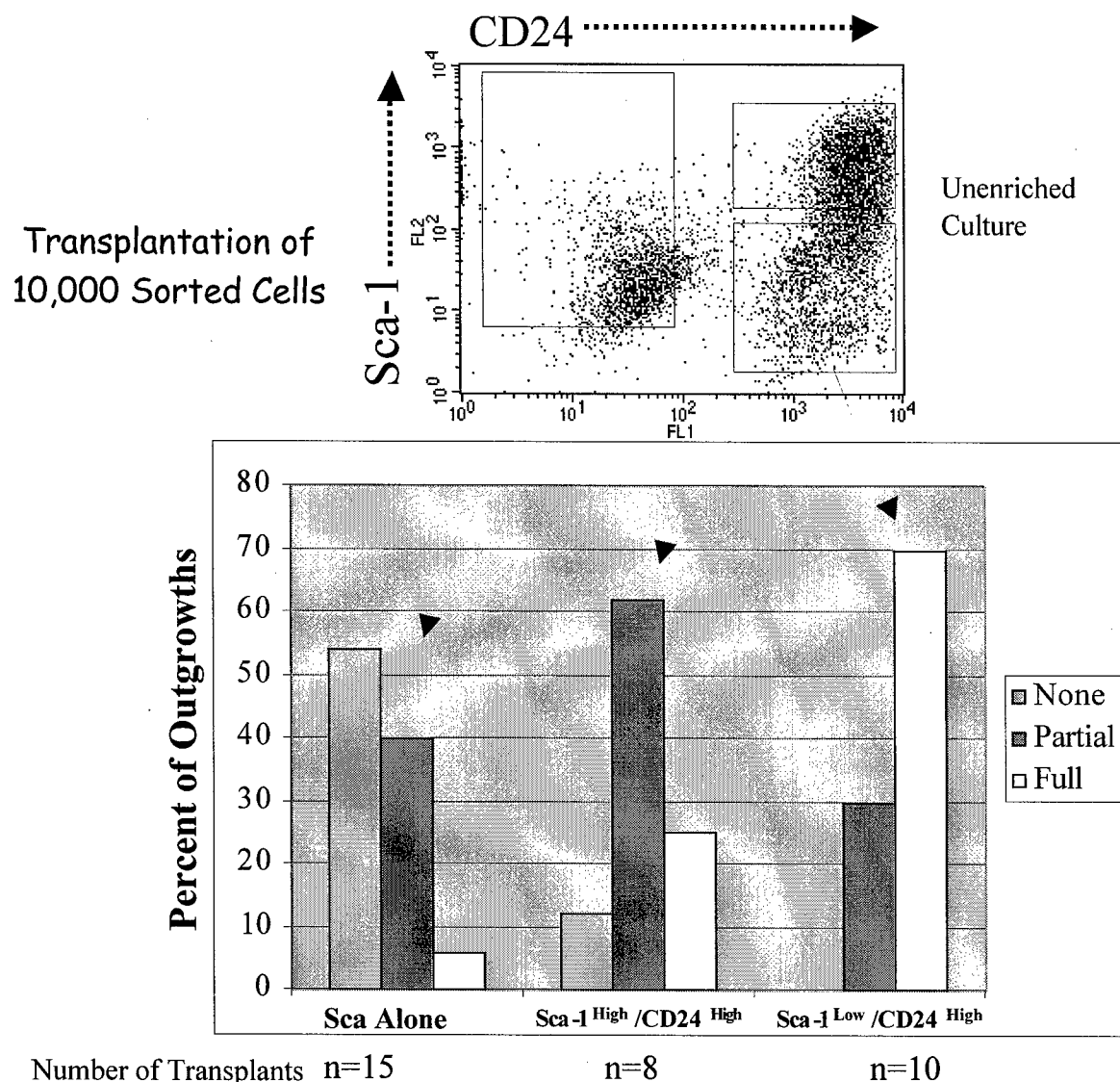
Figure 2. Cultured primary MECs isolated from wildtype mice and grown on plastic display three populations of cells as observed by Sca-1 and CD24 antibodies (top scatter plot). These populations include Sca-1 alone (about 30% of the cells), Sca-1 ^{high} / CD24 ^{high} (about 45%) and Sca-1 ^{low} / CD24 ^{high} (about 15%). These cell populations were isolated by FACS and 10,000 cells of each population were transplanted into cleared fat pads of syngeneic mice. The transplants were wholemounted after 8 weeks of growth and the outgrowth was qualified as either no outgrowth (None), partial outgrowth (Partial) and full outgrowth (Full) based on the relative area of fat pad occupied (bottom panel). The best outgrowths were observed from the Sca-1 ^{low} / CD24 ^{high} cells.

Cultured Primary MECs can be Enriched for Increased Outgrowth Capacity

Depleted Culture   Enriched Culture
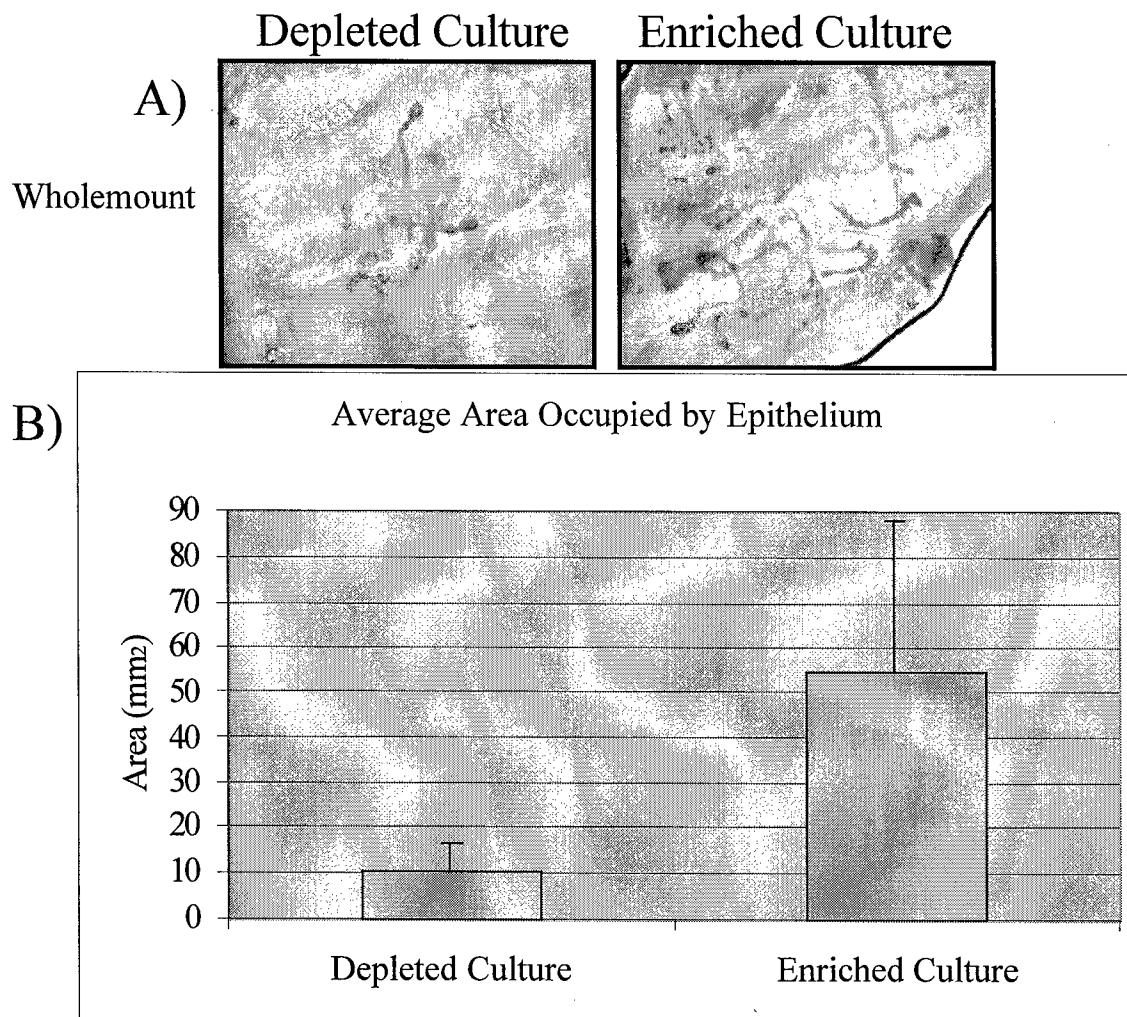
A)

Wholemount

B)

Figure 3. Two cultured populations were isolated from primary MECs. Both populations (depleted and enriched cultures) were transplanted into syngeneic mice to determine which possessed the best outgrowth capacity. We transplanted 50,000 cells into cleared fat pads and let the cells grow for 4 weeks (A). The enriched culture produced outgrowths that covered an area that was greater than 5 times more than the depleted culture cells(B).

The "Enriched Culture" Primary MECs are Sca-1^{Low}/CD24^{High}

No Enrichment

Depleted Culture
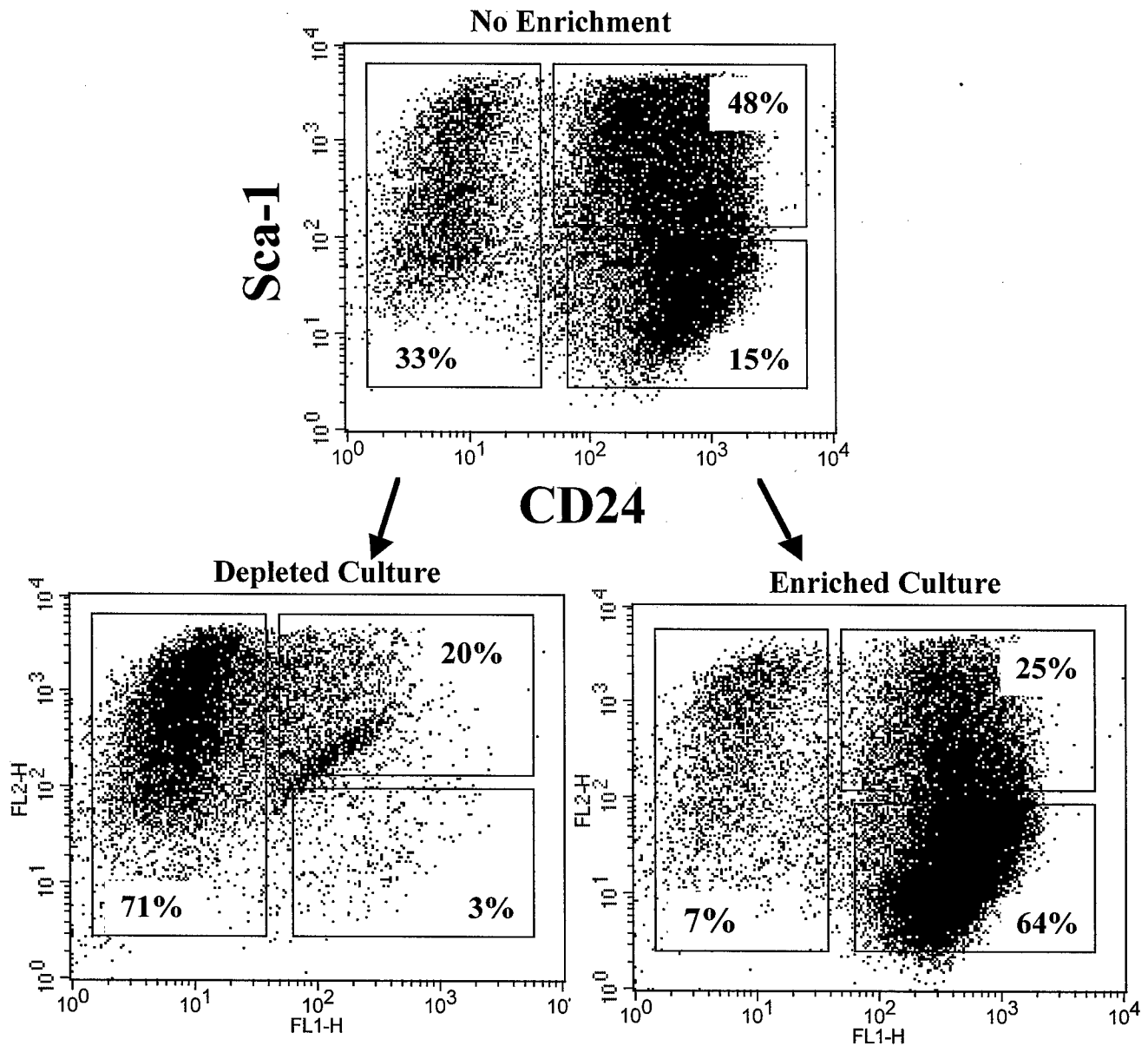
Enriched Culture

Figure 4. Unenriched primary MECs cultured on plastic have three distinct populations of cells as observed by FACS using Sca-1 and CD24 antibodies (top scatter plot). These populations include Sca-1 alone (about 30% of the cells), Sca-1^{high} / CD24^{high} (about 45%) and Sca-1^{low} / CD24^{high} (about 15%). The enriched primary MEC culture(bottom right scatter plot) has a four fold increase in the Sca-1^{low} / CD24^{high} cells and the depleted culture (bottom left scatter plot) has a five fold reduction of these cells as compared to the unenriched culture.

Limited Dilution Transplantation of Tumor Cells

Wnt1 total		
# cells injected	tumor formation	percentage
500,000	4/4	100
100,000	4/4	100
10,000	3/4	75
1,000	0/5	0
100	0/4	0

PyMT total		
# cells injected	tumor formation	percentage
100,000	2/2	100
25,000	2/2	100
10,000	3/4	75
5,000	7/9	77
1,000	11/18	61
200	4/12	33
100	1/5	20
20	0/15	0

Figure 5. Cells isolated from MMTV-Wnt-1 and MMTV-PyMT tumors were transplanted into the cleared fat pad of syngeneic mice at limiting dilutions. In our first round of transplantation of MMTV-Wnt-1 cells it was observed that between 1,000 and 10,000 cells were required to form a tumor. A second round of transplantation is in progress to further define the number of MMTV-Wnt-1 induced tumor cells required to form tumors. In contrast to the MMTV-Wnt-1 results it was observed that only 100 cells from MMTV-PyMT induced tumors are required to form tumors upon transplantation. Some of these data are from a collaboration with A. Welm and .M. Bishop.

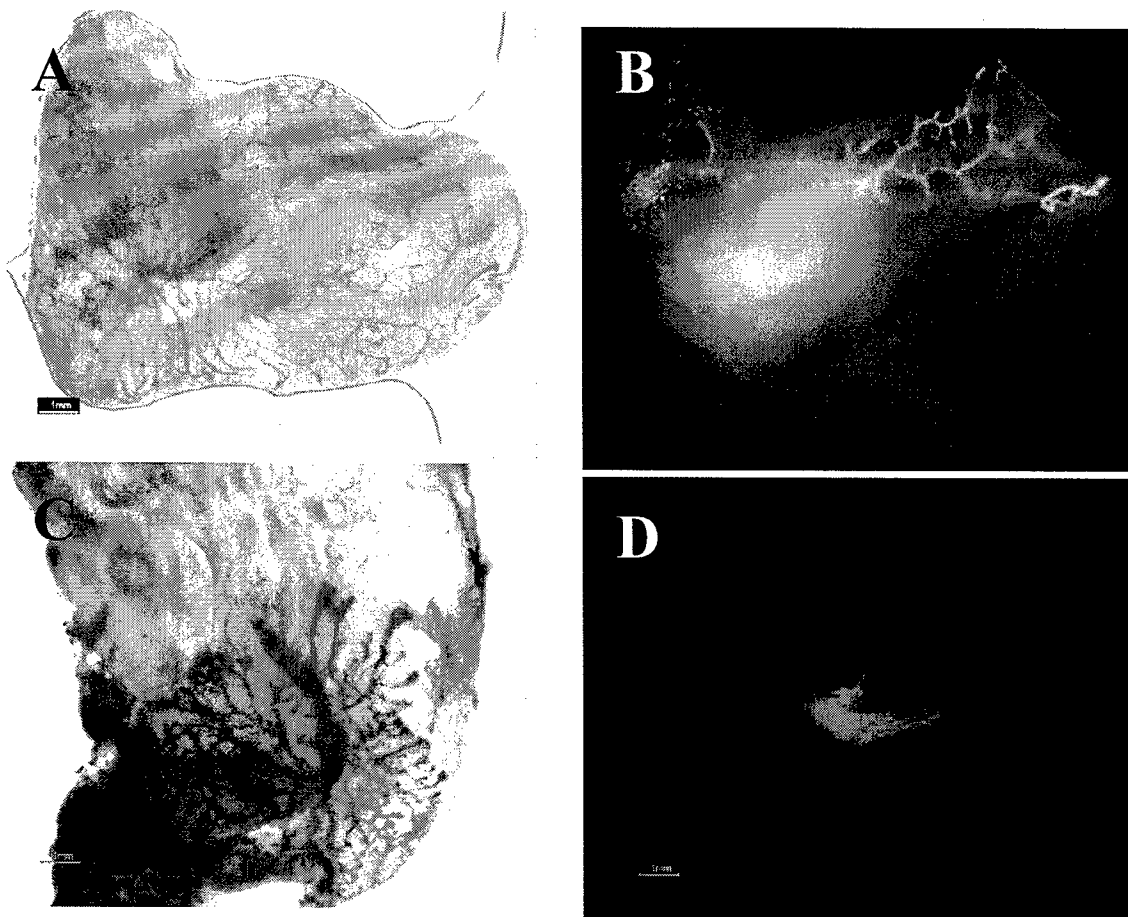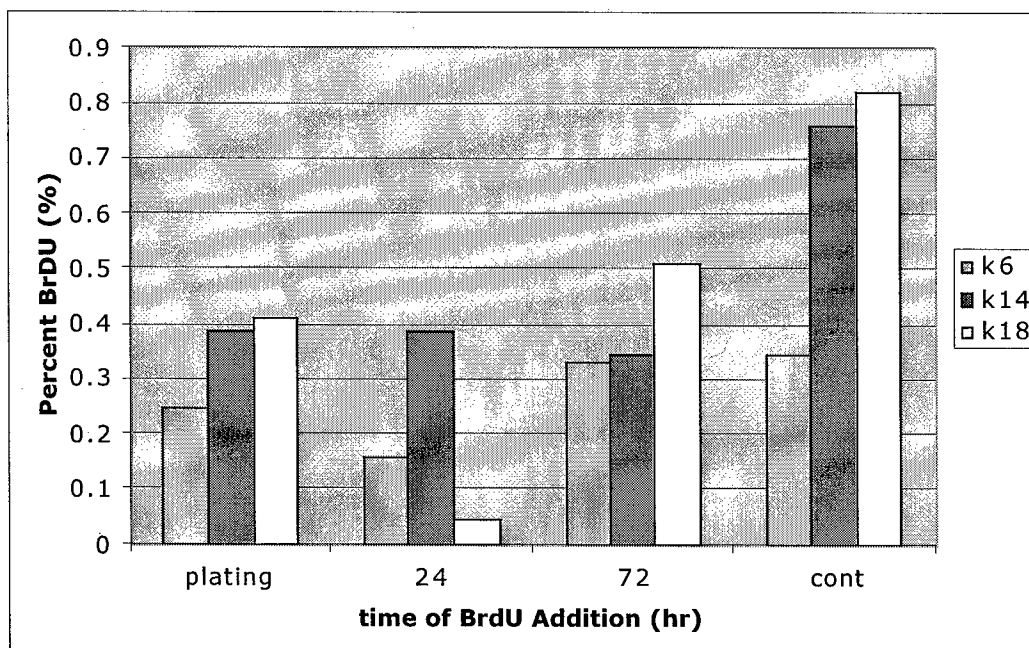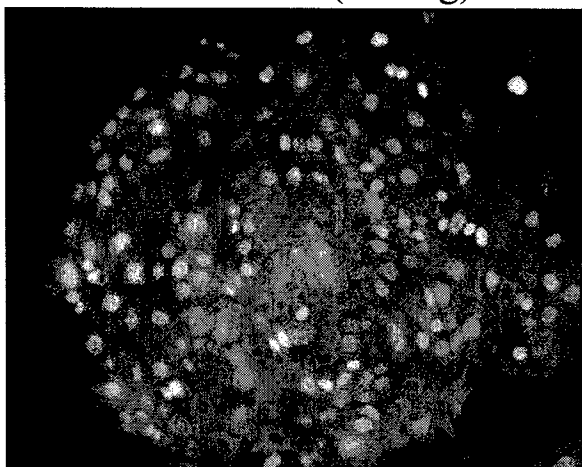# Retroviral based Infection and Transplantation of Primary MECs

Figure 6. Cultured primary MECs were infected two times with a ZsGreen expressing retrovirus using a centrifugation based "spin"infection published by A. Welm, *et. al*. Using this method we observed that around 20% of the primary culture cells expressed ZsGreen prior to transplantation. These cells were transplanted into cleared fat pads and allowed to grow for 8 weeks. We observed that about 20% of the transplants contain some ZsGreen positive outgrowths. However,in each positive outgrowth only about 10-20% of the ducts were positive. Panels A and C are carmine stained wholemounts and panels B and D are fluorescent images of outgrowths. Panels A and B are images of the same outgrowth. Panel B shows the most ZsGreen positive ducts that we observed in a transplant using the spin infection method. Panels C and D are the same outgrowth and are more representative of amount of fluorescence in a positive outgrowth.

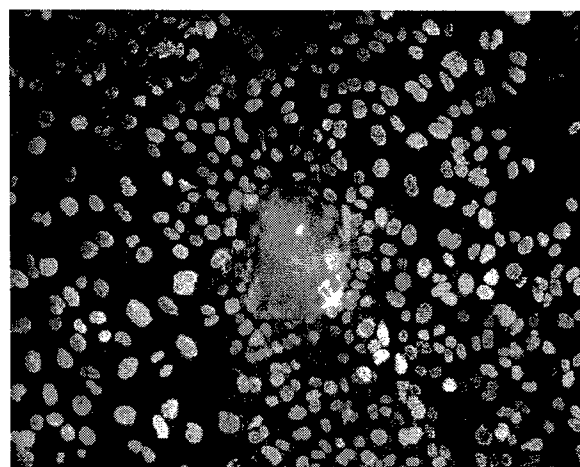# Proliferation of Cultured Primary MECs

Figure 7. Cultured primary MECs are highly proliferative. Since retrovirus infection requires proliferation for integration of viral DNA into the cell's genome we analyzed the amount of proliferation in cultured primary MECs. These experiments allowed us to determine the best time for retroviral infection of the MECs. Cells were treated with BrdU at the time indicated (top panel) to determine the amount of proliferation at each timepoint. For plating (attachment), cells were treated with BrdU during attachment of the cells to the plate. A continuous dose of BrdU for 96 hours was also given to the cells (cont.) to determine if any cells were quiescent culturing. The percent keratin-14 (k14), keratin-6 (k6) or keratin-18 (k18) cells that were also positive for BrdU is shown in the graph (top panel). The bottom panels show keratin-18 (red) and BrdU (green) staining of the primary cultures during attachment and 72 hours after plating. The method for producing primary MECs results in >90% of the cells K18 positive.

High Efficiency of the Optimized Infection Method
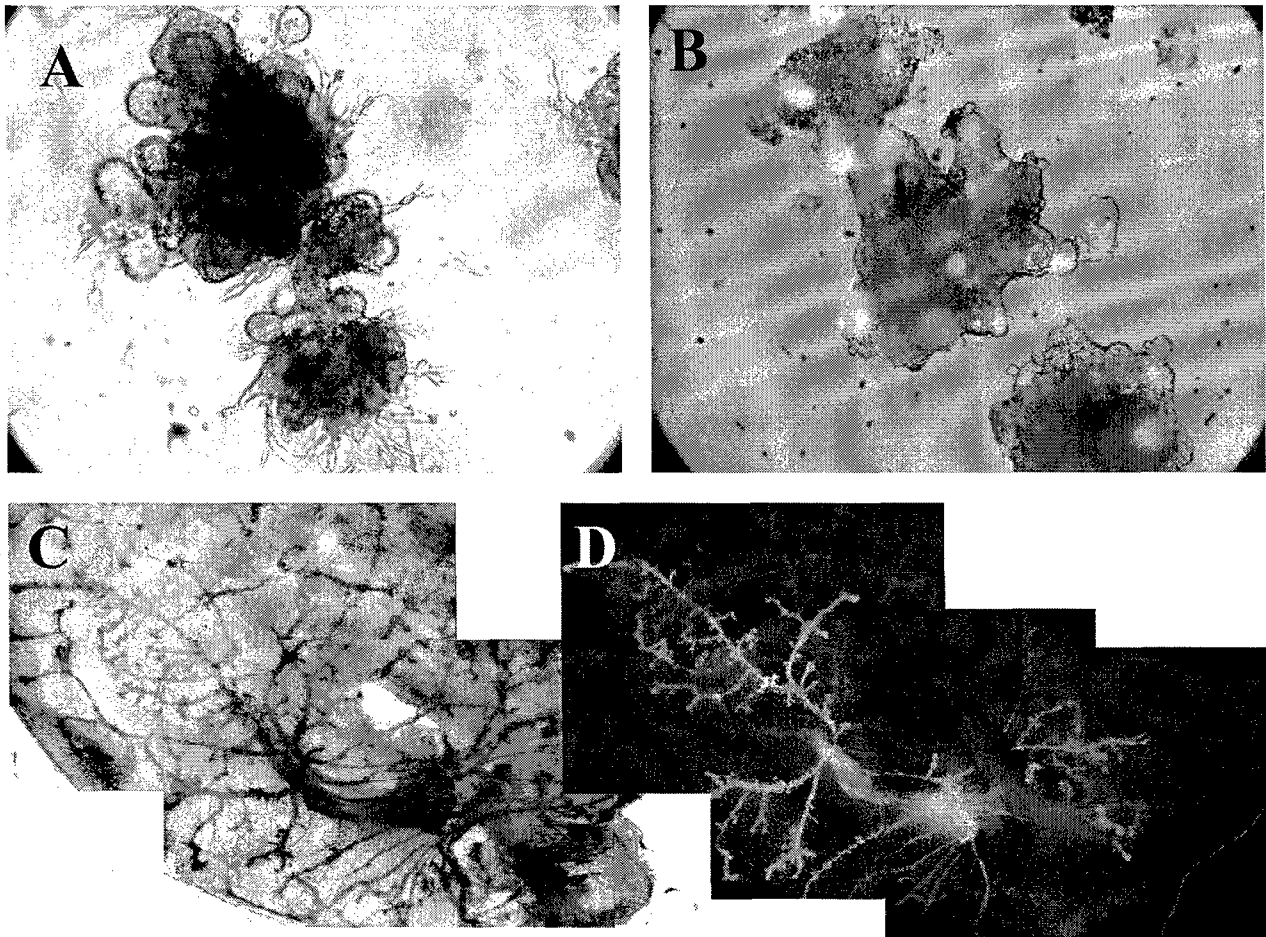

Figure 8. Primary MECs were cultured on plastic and processed to enrich for cells with enhanced outgrowth capacity. These cells were able to branch in matrigel and were efficiently transduced with EGFP expressing viruses (A and B). Enriched primary MECs were infected and transplanted into cleared fat pads and grown for 4 weeks. All transplants from these infected primary MECs were EGFP positive and 80-100% of the ductal epithelium in each outgrowth displayed green fluorescence (C and D). Panels C and D are the same outgrowth. Panel C is a carmine stained wholemount and panel D is an image of fluorescence.
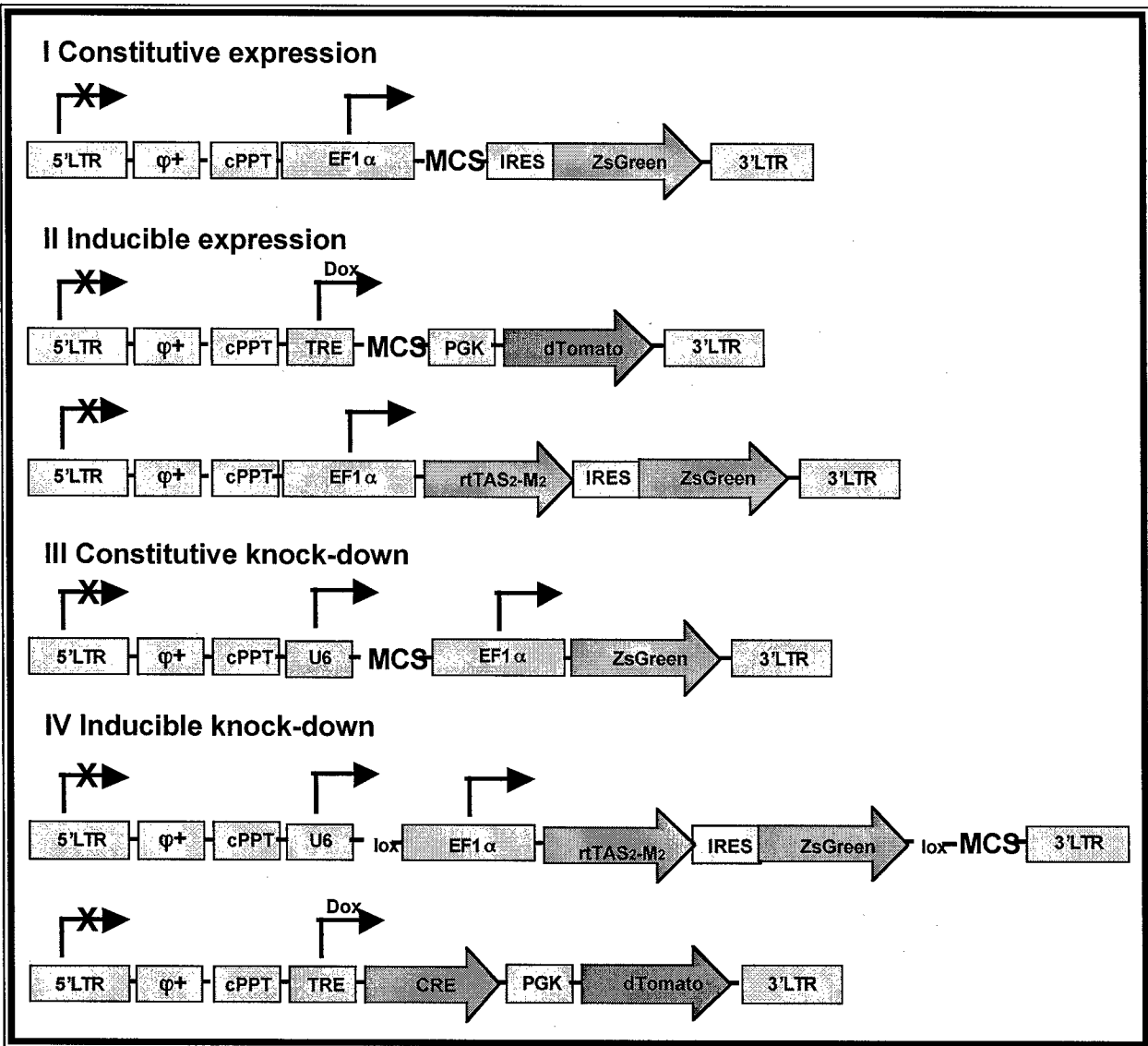
Figure 9. Lentiviral transfer vectors used in this study. 5 'LTR; 5' long terminal repeat. φ+; extended packaging signal, includes RRE (Rev-response element) which enables nucleo - cytoplasmic transport of the viral mRNA. cPPT; central polypurine tract, improves nuclear translocation of the pre-integration complex. 3 'LTR; 3' long terminal repeat, provides the self-inactivating feature of the vector as it contains a deletion in the U3 enhancer region. All these elements are depicted in yellow and were derived of pSICO (Ventura *et al.*, 2004).

MET and *MYC* cooperate in mammary tumorigenesis

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In human breast cancer, overexpression of the protooncogene *MET* is strongly associated with poor prognosis and high risk of metastasis. It stands out as a reliable prognostic indicator of survival and defines a set of tumors exclusive of those that express *HER2* or hormone receptors. Studies have shown that overexpression of mutant forms of *MET* cause cancer in mice. However, *MET* mutations have not been found in human breast cancer, and the consequences of overexpression of normal *MET* are unknown. To investigate the role of *MET* and other putative oncogenes in breast cancer, we developed an experimental system that involves retroviral delivery of genes into primary mammary epithelial cells, followed by transplantation of the transduced cells into mammary fat pads. Using this approach, we found that overexpression of wild-type *MET* leads to the development of nonprogressive neoplasms. The lesions progressed to mammary adenocarcinoma when a second protooncogene, *MYC*, was overexpressed, indicating that *MET* and *MYC* cooperate in mammary tumorigenesis. Both the nonprogressive neoplasms and adenocarcinomas display characteristics consistent with transformation and expansion of mammary progenitor cells. The approach described here should provide a useful model with which to efficiently test effects of various genes on tumor development in the breast.

breast cancer | mouse models | retrovirus

The *MET* protooncogene encodes a transmembrane receptor tyrosine kinase, Met, that has been widely implicated in tumorigenesis. Mutations that result in constitutive activation of Met are common in hereditary papillary renal carcinoma and have also been reported in hepatocellular carcinoma, gastric cancer, ovarian cancer, and squamous cell carcinoma. In addition, the wild-type *MET* gene is amplified or overexpressed in many other types of human cancer, including breast cancer (1). Several studies have found that high levels of Met are associated with poor prognosis and high risk of metastasis in breast cancer (2–4). Overexpression of Met occurs in a group of tumors distinct from those that express estrogen and progesterone receptors and those that overexpress *HER2* (5), suggesting that inhibition of Met may provide an opportunity to treat tumors for which no targeted therapy is available.

Little is known about the tumorigenic function of *MET in vivo*. Overexpression of constitutively activated *MET* mutants under the metallothionein-1 promoter led to the development of metastatic mammary tumors in transgenic mice (6, 7), and replacement of the wild-type *MET* allele with various *MET* mutants resulted in the development of several types of tumors (8). Although these results provide insight into the tumorigenic capabilities of mutant Met proteins, our goal was to generate a mouse model in which the effects of wild-type *MET* overexpression could be studied, because *MET* is not mutated in human breast cancer.

Existing mouse models of breast cancer exhibit significant differences in pathology and metastasis from the human disease (9). This may be partly attributed to different breast anatomy and physiology between the two species. It is also possible that the majority of genetically engineered mouse models, which target expression of oncogenes to the mammary gland under promoters that are preferentially expressed in differentiated cells, do not recapitulate spontaneous transformation of various cell types in

the human breast. Thus, mouse models in which different cell types can be targeted for transformation might prove useful. A recent report described that overexpression of certain oncogenes, but not others, results in expansion of the mouse mammary progenitor cell population, and this correlates with heterogenous tumor phenotypes (10). This phenomenon supports a current model for human breast cancer in which stem or progenitor cells become transformed, resulting in cellular heterogeneity within tumors as well as phenotypic diversity between tumors (11, 12). Effects of oncogene activation in mammary progenitor cells are difficult to examine, because mammary-specific promoters are highly responsive to steroid hormones (13, 14) and are highly expressed in more differentiated steroid receptor-positive cells. Specific targeting of transgene expression in mammary progenitor cells has not yet been described.

In the developing mammary gland, stem and progenitor cells are present within terminal end buds and along mature ducts and give rise to both myoepithelial and luminal epithelial lineages (15–17). Molecular characterization of mammary progenitor cells has revealed that they are contained within a population of cells that express Sca-1 (15). However, ≈20% of mammary epithelial cells (MEC) are Sca-1-positive, indicating that not all Sca-1-positive cells are progenitor cells. A second more restricted marker of these progenitor cells is cytokeratin 6 (CK6). CK6-positive cells are contained within the Sca-1-positive population (10). CK6 is normally expressed in the mammary gland during embryonic stages and within terminal end buds during ductal morphogenesis, but CK6-positive cells are rare in mature differentiated glands (18).

To better understand the contribution of genes such as *MET* to breast cancer without restricting expression to differentiated cells, we optimized a retroviral system to introduce genes of interest into the mammary gland with a vector that was likely to express *MET* in stem and/or progenitor cells. Using this system, we found that overexpression of *MET* resulted in the development of multiple microscopic foci of neoplasia that failed to progress to full-blown malignancies. Although overexpression of *MET* alone did not result in the development of tumors, we found that an additional genetic stimulus, overexpression of *MYC*, cooperated with *MET* in mammary tumorigenesis. Overexpression of *MYC* alone resulted in epithelial hyperplasia as previously reported in studies of transgenic mice (19–21). The nonprogressive neoplasia (NPN) and tumors resulting from *MET* and *MET/MYC* overexpression, respectively, displayed cellular markers characteristic of mammary progenitor cells, suggesting that *MET* may play an important role in transformation and expansion of these cells.

Materials and Methods

Transgenic Mice. Mouse mammary tumor virus (MMTV)-rtTA transgenic mice were obtained from Lewis Chodosh (University of Pennsylvania School of Medicine, Philadelphia) (22). TRE-

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MET transgenic mice were described previously (23). MMTV-rtTA/TRE-*MET* mice were maintained on chow containing 200 mg/kg doxycycline to induce transgene expression.

Preparation of Ecotropic Retrovirus. BOSC-23 cells were cultured in DMEM with glutamine and 10% FBS. To prepare virus, the cells were cotransfected with pCL-Eco plasmid (24) and the pMIG retroviral vector, which was obtained from Yosef Refaeli (National Jewish Medical and Research Center, Denver) (25). One day after transfection, the medium was changed, and collection of virus-containing medium began on day 2. The medium was filtered through a 0.45-μm filter. Collection of virus from the same cells continued for 1 more day.

Preparation of Primary Mouse MEC. For preparation of primary mouse MEC, 10- to 12-wk-old donor mice were killed, and only the nos. 3, 4, and 5 gland pairs were harvested to minimize contamination with muscle and other tissues. Inguinal lymph nodes were removed before isolation of the no. 4 glands. MEC cultures were prepared as described (26), except that glands were minced with razor blades for 5 min and then digested in 5 ml of collagenase buffer (RPMI with 10 mM Hepes/2.5% FBS/100 μg/ml penicillin/streptomycin) per gram of tissue in the presence of collagenase (1 mg/ml, Sigma) for 1 h at 37°C while shaking at 200 rpm. The cells, still aggregated as mammary organoid structures, were washed five times with collagenase buffer, including two rapid-pulse centrifugation steps to remove single cells such as fibroblasts and enrich for large mammary organoids, as described (27). Organoids were pooled and plated over an area of 45–90 cm^2 per gram of tissue (weighed before collagenase treatment) in six-well tissue culture plates (Corning). The organoids were plated in MEC medium [50% DMEM with glutamine/50% F-12 with glutamine/10 mM Hepes/10% FBS/5 μg/ml insulin/1 μg/ml hydrocortisone (Sigma)/10 ng/ml recombinant epidermal growth factor (Roche Applied Science)/50 μg/ml gentamicin/100 μg/ml penicillin/streptomycin] and allowed to attach overnight.

Optimization of Retroviral Infections. Analysis of factors that contribute to successful infection of primary MEC was carried out by using GFP as a marker of infected cells and systematically altering the following parameters: the method used to isolate MEC, cell density during infection, the presence of various growth factors in the medium, retroviral packaging cell lines, polybrene supplementation, length and speed of centrifugation during infection, and the number of successive infections. Factors that proved most important for successful infection of primary MEC are the following: (*i*) glands should not be overly minced before treatment with collagenase; (*ii*) to produce the highest viral titer, BOSC-23 cells should be transfected with additional plasmid encoding viral packaging proteins (vs. BOSC-23 cells alone or 293T cells transfected with the packaging proteins); and (*iii*) polybrene concentration should not exceed 1 μg/ml (data not shown). Using these conditions, we obtained 25–65% of cells expressing GFP after two successive infections, measured by FACS analysis (Fig. 6*B*, which is published as supporting information on the PNAS web site). We found no significant improvement in infection efficiency when a third spin infection was carried out (data not shown). Cells were infected on 2 consecutive days by replacing the MEC medium with BOSC-23 medium containing packaged retrovirus and supplemented with polybrene (1 μg/ml, Sigma). The plates were centrifuged at room temperature for 1 h at 600 × g. The conditioned medium was then discarded, and fresh MEC medium was added to the cells.

Transplantation of Infected MEC into Cleared Mammary Fat Pads. On the fourth day in culture, MEC were trypsinized to a single cell suspension, washed with PBS, and counted. The cells were resuspended at a concentration of 10^8 cells per ml in PBS and transferred to ice. Three-week-old recipient mice were anesthetized with avertin (125–400 mg/kg), and 10^6 MEC were injected in a volume of 10 μl into the cleared inguinal fat pads, as described (28).

Whole Mounts and Tissue Processing. Glands were dissected from mice and flattened between two glass slides for visualization of GFP expression on a fluorescent dissecting microscope. The glands were then fixed in 4% paraformaldehyde on ice for 4–16 h. Carmine alum staining of whole mounts and processing of tissue for histology was performed by using standard methods.

Immunohistochemistry. Tissue sections were stained by using ABC Elite and MOM immunohistochemistry reagents and avidin/biotin blocking reagents (Vector Laboratories). Antibodies used were anti-cytokeratin 18 (1:30; Progen, Heidelberg), anti-cytokeratin 14 (1:10,000; Covance, Princeton), anti-CK6 (1:100; Covance), antiphospho-tyrosine-Met (1:25; Cell Signaling Technology, Beverly, MA), and anti-human Met (1:500; Zymed).

Results

Overexpression of *MET* via Retroviral Transduction in Primary MEC Resulted in Focal NPN. To determine whether overexpression of wild-type *MET* contributes to the development of breast cancer, we took two approaches. First, we obtained transgenic mice that express the tetracycline-responsive transactivator protein under the MMTV promoter (MMTV-rtTA) and crossed them to transgenic mice that express *MET* under the tetracycline response element (TRE-*MET*). Despite extensive analysis, we could not discern any consistent adverse effect of *MET* overexpression in MMTV-rtTA/TRE-*MET* animals, even though *MET* was appropriately overexpressed, and the protein was activated by phosphorylation (Fig. 7, which is published as supporting information on the PNAS web site).

As a second approach to overexpress *MET*, we devised a system in which we hoped to target gene expression to multiple cell types in the mammary gland, including mammary progenitor cells. We infected primary MEC with pMIG, a retroviral vector derived from mouse stem cell virus, which was originally developed for its ability to be expressed in embryonic stem cells (29). We optimized retroviral infection of MEC, as described in *Materials and Methods*. Using an ecotropic envelope, we were able to consistently achieve infection of 25–65% of primary MEC, as determined by FACS analysis using GFP as a marker of infected cells (Fig. 6*B*). After infection, the MEC were transplanted into cleared inguinal fat pads of 21-day-old syngenic mice. Reconstituted glands harvested 8–10 wk after transplantation showed normal outgrowth of GFP$^+$ cells and, as expected, all fat pads were filled with ductal structures arising solely from the transplanted cells; control fat pads that were cleared of endogenous epithelium but did not receive injections of cells remained empty (data not shown). Because the transplanted MEC gave rise to GFP$^+$ outgrowths, a portion of the infected cells were indeed mammary progenitor cells; only progenitor cells are capable of giving rise to a mammary outgrowth after transplantation (15, 30).

To conditionally overexpress wild-type human *MET* in the mouse mammary gland, we infected primary MEC from mature virgin TRE-*MET* female mice with a retrovirus expressing a cassette consisting of the tetracycline-repressible transactivator protein (tTA) followed by an internal ribosome entry site and GFP (pMIG-tTA; diagrammed in Fig. 6*A*). As controls, TRE-*MET* transgenic MEC were infected with empty pMIG vector, which expresses GFP alone (25). Animals were treated with doxycycline to repress expression of *MET* or were left untreated to allow for *MET* overexpression.
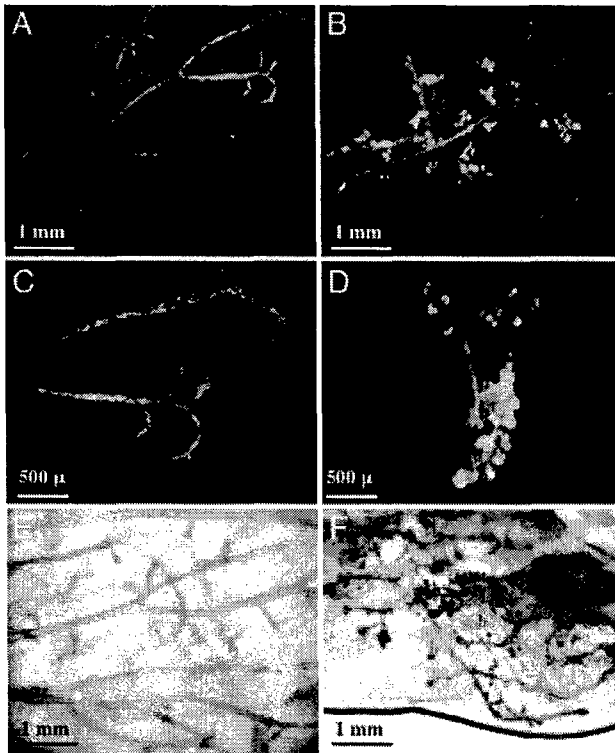
Fig. 1. Overexpression of wild-type *MET* resulted in abnormal mammary gland development. Mammary gland outgrowths expressing either GFP alone (*A*, *C*, and *E*) or *MET* and GFP (*B*, *D*, and *F*) were evaluated by whole-mount microscopy 10 wk after transplantation. Infected cells were visualized by GFP fluorescence (*A–D*), and the entire gland was visualized by carmine alum staining (*E* and *F*).

Fig. 2. Overexpression of *MET* resulted in ductal filling and loss of organization. Glands expressing GFP (*A*, *C*, and *E*) or *MET* and GFP (*B*, *D*, and *F*) were sectioned and stained with hematoxylin/eosin (*A* and *B*). Alternatively, sections were stained with antibodies specific for cytokeratin 18 to visualize luminal epithelial cells (*C* and *D*) or with antibodies against cytokeratin 14 to detect myoepithelial cells (*E* and *F*). Sections from mice that received MEC in which *MET* expression was repressed by treatment with doxycycline (*G*) or from mice that received MEC overexpressing *MET* (*H*) were stained with antibodies specific for tyrosine-phosphorylated Met.

Ten weeks after transplantation, mammary gland outgrowths that overexpressed *MET* displayed abnormal lobule-like structures (Fig. 1 *B*, *D*, and *F*). Glands arising from MEC expressing GFP alone had no abnormalities (see Figs. 1 *A*, *C*, and *E* and 2 *A*, *C*, and *E*). Hematoxylin/eosin staining of sections from mammary gland outgrowths revealed that the "lobules" in *MET*-expressing glands were actually disorganized clusters of epithelial cells (Fig. 2*B*). Immunohistochemical staining showed that the cytokeratin 18-positive luminal epithelial cells were abnormally clustered together, and often there was little or no discernable lumen (Fig. 2*D*). Likewise, the cytokeratin 14-positive myoepithelial cells were either scattered about or organized around filled islands of cells (Fig. 2*F*). Histological analysis confirmed that the epithelial cells were atypical with large nuclei, condensed chromatin, large nucleoli, and little cytoplasm (data not shown). TRE-*MET*/pMIG-tTA mammary outgrowths from mice that were treated with doxycycline to repress expression of *MET* were not distinguishable from those expressing GFP alone (Fig. 2*G* and data not shown).

Based on accepted nomenclature according to the Annapolis guidelines (www.nih.gov/Annapolis-guidelines) for mouse mammary pathology, the phenotype induced by overexpression of *MET* most closely resembles mammary intraepithelial neoplasia, a spectrum of intraluminal epithelial proliferations with cytologic atypia that includes *in situ* carcinomas (31). However, many of the lesions resulting from overexpression of *MET* do not have an intact myoepithelial layer, indicating the neoplasias are not strictly intraluminal. Because the lesions do not progress to tumors (see below), we refer to the *MET*-induced lesions as NPN. A total of 26 glands expressing *MET* were analyzed in five independent experiments. Multiple microscopic neoplastic foci
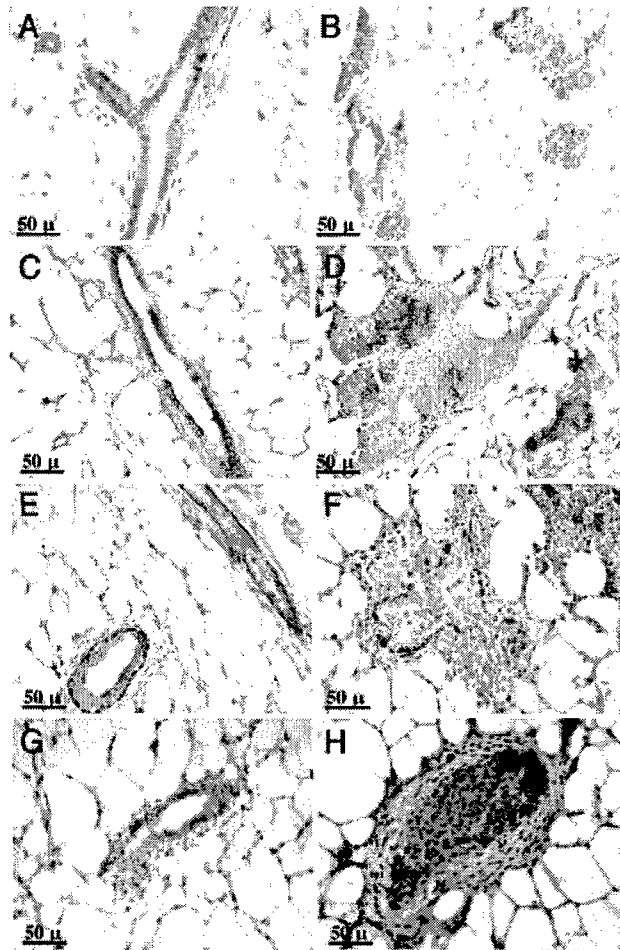
were observed in ≈77% (20/26) of the glands after successful transplantation. No green cells were detected in 6/26 of the glands despite successful mammary reconstitution. An additional nine glands were analyzed from mice maintained on doxycycline to repress *MET* expression. All nine glands appeared normal (as shown in Fig. 2*G*).

Immunohistochemical staining with antibodies specific for Met (not shown) and tyrosine-phosphorylated Met (Fig. 2*H*) verified that Met protein was present and actively signaling in epithelial cells that filled the ducts but not in the surrounding stroma. There was evidence for an inflammatory response, manifested by the presence of increased numbers of CD45+ leukocytes compared to the control outgrowths (data not shown); however, these cells represented only a small portion of the stroma in *MET*-expressing outgrowths. Our data show that overexpression of wild-type *MET* in the mouse mammary gland leads to NPN with loss of ductal organization. The phenotype was observed by 8 wk after transplantation but did not progress; no tumors or noticeable worsening of the neoplastic lesions was detected over a period of 8 months.

To rule out the possibility that the observations made with

overexpression of *MET* via retroviral transduction/transplantation were an artifact of the procedure, we tested whether MEC expressing *MET* under control of the MMTV promoter (which has no effect in the intact mammary gland; Fig. 7) would develop NPN upon infection and transplantation. For these experiments, we isolated MEC from 10- to 12-wk-old MMTV-rtTA/TRE-*MET* transgenic mice, infected them with pMIG, and transplanted them into cleared fat pads of 21-day-old mice that were treated with doxycycline throughout the experiment to induce *MET* expression. Ten weeks after transplantation, the resulting glands did not show any abnormalities by fluorescent whole-mount analysis (data not shown). These results show that the retroviral infection/transplantation technique does not promote abnormal ductal outgrowth.

MET and MYC Cooperate in Mammary Gland Tumorigenesis. Because mammary outgrowths expressing *MET* via retroviral transduction do not progress to tumors over the course of several months, we speculated that the transplants might represent an ideal model in which to directly test cooperation of *MET* with other oncogenes in tumor progression. It has been demonstrated that multiparous transgenic mice expressing the ligand for Met, hepatocyte growth factor/scatter factor develop mammary tumors with an average latency of 8 months, and among other alterations, the tumors were shown to contain excessive amounts of activated Myc protein (32). *MYC* has been implicated in many types of human cancer, and it is overexpressed in a subset of breast cancers (33). Although *MET* and *MYC* loci are coamplified in some gastric cancers (34, 35), it is not known whether *MET* and *MYC* are cooverexpressed or whether these genes cooperate in the genesis of breast cancer.

To test whether overexpression of *MYC* would cause the *MET*-induced NPN to advance to tumors, we infected MEC from TRE-*MET* transgenic mice with pMIG-tTA alone or pMIG-tTA and pMIG-*MYC*. Alternatively, we infected MEC from nontransgenic mice with pMIG-*MYC* alone. Eight weeks later, we examined the mammary outgrowths. Although *MET* expression alone induced NPN (Fig. 3 *A–C*), expression of *MYC* alone caused increased branching and hyperplastic lateral budding (Fig. 3 *D–F*), as previously described in transgenic mouse models (19, 21). Coexpression of *MET* and *MYC* resulted in high-grade neoplasia in which ductal organization was no longer recognizable by 8 wk after transplantation (Fig. 3 *G–I*). Glands expressing both *MET* and *MYC* displayed a highly disorganized clustering of cells, demonstrated by staining for cytokeratin 14 (Fig. 3 *C, F,* and *I*). Whereas myoepithelial cells could still be detected surrounding ductal structures in outgrowths overexpressing *MYC* alone (Fig. 3*F*), outgrowths expressing either *MET* alone (Fig. 3*C*) or *MET* and *MYC* (Fig. 3*I*) were no longer organized and often did not contain an intact myoepithelial cell layer.

Although no tumors were observed in glands expressing either *MET* or *MYC* alone, mammary glands coexpressing *MET* and *MYC* progressed to focal palpable tumors by 10 wk. Histological analysis of tumors at 4 months revealed mammary adenocarcinoma (Fig. 4*A*), with features typical of highly proliferating lesions including prominent nucleoli and high mitotic indices (Fig. 4*B*). A total of 25 glands in two independent experiments were analyzed for cooperation between *MET* and *MYC*. Tumor penetrance was 100% after successful transplantation. An additional 17 glands were analyzed from mice that received doxycycline to repress *MET* expression (*MYC* was expressed alone). All of these glands developed various degrees of hyperplasia, as shown in Fig. 3 *D–F*.

Overexpression of *MET* in Neoplasms Correlates with an Abundance of the Early Developmental Marker CK6. Tumors resulting from overexpression of *MET* and *MYC* were remarkable in that they contained cells from both myoepithelial and luminal epithelial
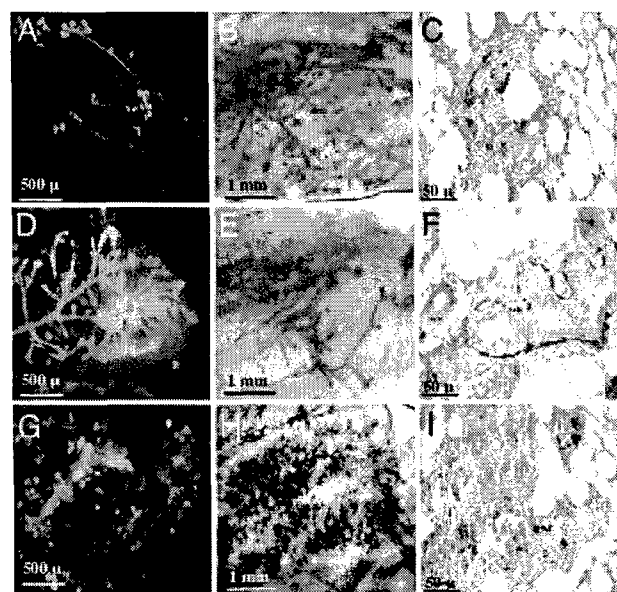

Fig. 3. *MET* cooperates with *MYC* to promote progression of neoplasia in the mammary gland. Whole mounts of mammary gland outgrowths harvested 8 wk after transplantation were viewed by fluorescent microscopy (*A, D,* and *G*) and by carmine alum staining and light microscopy (*B, E,* and *H*). Sections from mammary glands isolated at this stage were also stained with antibodies against the myoepithelial marker cytokeratin 14 (*C, F,* and *I*). Glands that expressed *MET* alone (*A–C*), *MYC* alone (*D–F*), or *MET* and *MYC* (*G–I*) are shown.

cell lineages, as illustrated by staining for cytokeratins 14 and 18, respectively (Fig. 4 *C* and *D*). A recent report revealed that mammary hyperplasias and tumors that are composed of heterogenous cell types also express early progenitor cell markers such as Sca-1 and CK6 (10). To determine whether tumors arising from *MET/MYC* overexpression might be a result of progenitor cell expansion, we examined the expression of CK6 by immunohistochemistry. Fig. 5*A* shows that *MET/MYC*-induced tumors contained numerous CK6-positive cells and thus fit into a class of tumors in which heterogeneity of cell types correlates with aberrant expression of the early developmental marker CK6.
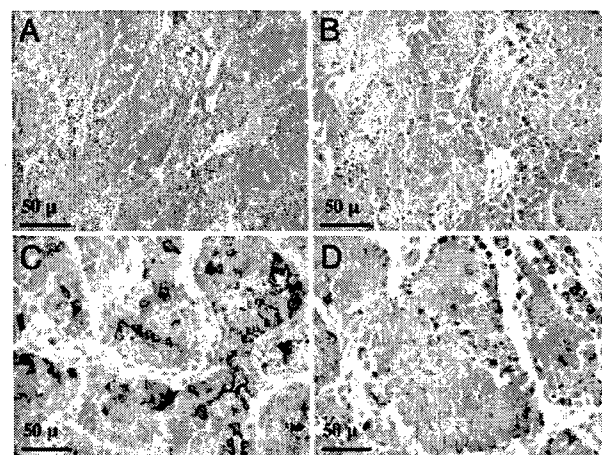

Fig. 4. *MET* and *MYC* cooperate in mammary tumorigenesis. Sections of tumors resulting from expression of both *MET* and *MYC* were stained with hematoxylin/eosin (*A* and *B*). The tumors were isolated 4 months posttransplantation. Sections were also stained with antibodies against cytokeratin 14 (*C*) and cytokeratin 18 (*D*).
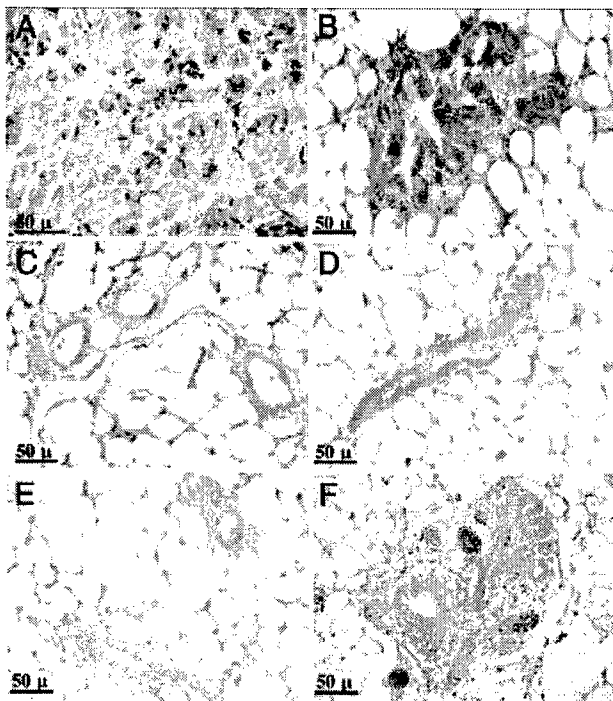
Fig. 5. Neoplastic lesions induced by *MET* express the mammary progenitor cell marker CK6. Immunohistochemistry with antibodies specific for CK6 was carried out on sections from the following samples: adenocarcinoma harvested 4 months after coexpression of *MET* and *MYC* (*A*), *MET*-induced neoplasia harvested 10 wk after transplantation (*B*), 10-wk mammary outgrowths that expressed GFP alone (*C*), and mammary glands from 10-wk-old virgin wild-type (*D*) or MMTV-rtTA/TRE-*MET* (*E*) mice. A mammary gland from a 1-year-old MMTV-rtTA/TRE-*MET* mouse containing a rare focal hyperplasia is shown in *F*.

Because *MET/MYC* tumors display characteristics consistent with expansion of a progenitor cell population, we investigated whether NPN might be the result of *MET* overexpression in progenitor cells. We found that lesions resulting from overexpression of *MET* contained large numbers of CK6-positive cells (Fig. 5*B*), whereas mammary outgrowths expressing only GFP and mammary glands from unmanipulated mice contained few or no CK6-positive cells (Fig. 5 *C* and *D*). Mammary glands from most MMTV-rtTA/TRE-*MET* transgenic mice also had virtually no CK6-positive cells and were not distinguishable from wild-type controls (Fig. 5*E*). Rare hyperplasias observed in MMTV-rtTA/TRE-*MET* mice >1 year of age were CK6-positive (Fig. 5*F*). Because *MET* is overexpressed and activated in glands from all MMTV-rtTA/TRE-*MET* mice (Fig. 7), we conclude that expression of CK6 is not merely a result of *MET* activity.

Discussion

Development of Breast Cancer Models Using Ecotropic Retroviral Infection and Transplantation of Primary MEC. We report the optimization of a system to efficiently overexpress genes of interest in the mouse mammary gland. This method has allowed us to generate mouse models of breast cancer that are different from the commonly used transgenic models. Overexpression of wild-type *MET* using our system resulted in multiple microscopic foci of neoplasia that did not progress to malignancy in the time frame of our experiments (>8 months) unless *MYC* was also overepxressed. This model should be useful to study progression of cancer and for the identification of additional oncogenic insults that can cooperate with *MET* in tumorigenesis.

In contrast to the results obtained by overexpression of wild-type MET into MEC via retroviral transduction and transplantation, overexpression of wild-type *MET* under the MMTV promoter did not result in abnormal mammary glands. It is formally possible that the MMTV promoter does not elicit *MET* expression at the exact developmental time or at levels above a required threshold to achieve a neoplastic phenotype. Because *MET* overexpression and activation are readily detectable at similar time points in both systems by parallel immunohistochemistry, however, it is more likely that the difference in outcomes lies in the cell types in which the promoters are expressed. Overexpression of *MET* by the MMTV promoter may occur mainly in differentiated, hormone-responsive cells, and that may not result in cell transformation. In contrast, the retroviral vector used in the present work expresses in a variety of cell types (data not shown), including progenitor cells that give rise to mammary gland outgrowth (as in Fig. 1). Furthermore, repopulation of the mammary gland after transplantation of MEC requires progenitor cell expansion (15, 30), so mature outgrowths that overexpress *MET* presumably arose from progenitor cells overexpressing *MET*.

MET-Induced Neoplastic Lesions Display Characteristics of Mammary Progenitor Cells. Luminal epithelial cells and myoepithelial cells within the mammary gland are derived from a common progenitor (36, 37). Most mouse mammary tumors display a loss of cytokeratin 14-positive myoepithelial cells as the luminal epithelium expands. However, we found that tumors resulting from overexpression of *MET* and *MYC* in our system contained both luminal and myoepithelial cells, suggesting that the tumors might arise from an expanded population of bipotential mammary progenitor cells.

Expansion of mammary progenitor cells in this model is further supported by our finding that NPN initiated by overexpression of *MET* alone consisted of CK6-positive cells. An abundance of CK6-positive cells is thought to reflect expansion of a progenitor cell population (10). It has previously been found that expression of *MET* increases 5-fold during differentiation of human breast progenitor cells *in vitro* (37). Although *MET* expression may be regulated during differentiation of breast epithelium, the outcome of aberrant expression of *MET* in progenitor cells has not directly been tested.

Similar to our results, hyperplasias and tumors induced by Wnt-1, β-catenin, or Myc are heterogenous in nature and express both CK6 and Sca-1. On the other hand, hyperplasias and tumors driven by Neu, H-Ras, or the polyomavirus middle T antigen do not express CK6 or Sca-1 and are much more homogenous in nature (10). Because of the presence of early developmental markers and the coexistence of multiple epithelial cell lineages, the former group of tumors is thought to arise from expansion of a transformed progenitor cell population, whereas the latter group might arise from transformation of a more differentiated cell type. Thus far, tumors suspected to arise from a putative progenitor cell population all express oncogenes that lead to activation of components of the Wnt signaling pathway (10). Our data remain consistent with this: β-catenin was found in the cytoplasm and nuclei of cells within *MET*-induced NPN, indicating that it is active (Fig. 8, which is published as supporting information on the PNAS web site). Tumors arising in WAP-HGF transgenic animals also displayed activation of β-catenin (32), and *MET* has been implicated in β-catenin activation in other cell types as well (38, 39).

It is important to note that not all hyperplasias or tumors arising from the retroviral transduction/transplantation system express CK6. For example, we have found that tumors arising from expression of the Polyomavirus middle T antigen were CK6-negative whether the oncogene was expressed by retroviral transduction/transplantation (Fig. 9, which is published as sup-

porting information on the PNAS web site) or from the MMTV promoter (10). In contrast, hyperplasias resulting from overexpression of *MYC* were CK6-positive in both MMTV-*MYC* transgenic mice (10) and in outgrowths resulting from retroviral transduction of *MYC* (Fig. 9). Based on these results, it is likely that expression of an oncogene in progenitor cells (as with the retroviral transduction/transplant system) is not sufficient for generation of tumors that consist of an expanded progenitor cell population. Instead, transformation and expansion of progenitor cells may also require the function of particular oncogenes.

There is a clear association between CK6 expression and the Sca-1$^+$ mammary progenitor cell population: CK6$^+$ cells are present in the Sca-1$^+$ cell population and absent in the Sca-1$^-$ cell population (10). CK6 expression might be a better marker of mammary progenitor cells, because it is more restrictive than Sca-1 expression (10). Still, it should be noted that CK6$^+$ cells have not yet been proven to be bona fide progenitor cells that are capable of giving rise to multiple cell types in the mammary gland. This will require either identification of new progenitor cell markers in the mammary gland, or a method to sort viable cells based on expression of intracellular markers such as CK6 without permeating the cell membrane.

MET and Metastasis. Met activity has been implicated in cell migration and invasion, and there is a strong clinical association between *MET* overexpression and poor prognosis due to metastasis. We did not detect metastasis from *MET/MYC*-induced mouse mammary tumors (unpublished data), although our experimental system is capable of giving rise to metastatic tumors initiated by other protooncogenes (unpublished data). Our data suggest that, at least in mice, *MET* overexpression is not sufficient for tumor metastasis. We have not ruled out that *MET* plays a role in this process, perhaps in cooperation with other factors.

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